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The Regulation of Id2 Gene Expression in J774.2 Macrophages

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Dedicated to

KALAYA AND CHARIT TINGSABADH
and
KUMAREE CHALERMLUK CHITRABONGS

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Abstract

Id2 is a HLH factor with well established role in cellular proliferation and differentiation. Traditionally Id2 is classified as a dominant negative member of the HLH family, yet it shows broad association with proteins from other families. Changes in its expression in response to hormonal and growth signal differs greatly between cell types though little is known about its regulation in macrophages. In this study the regulation of Id2 protein expression by glucose and hormones is studied in J774.2 macrophages.

In J774.2 cells glucose induces increases in protein levels of Id2. Up regulation of Id2 requires glutamine, is mimicked by glucosamine and is inhibited by azaserine, an inhibitor of glutamine:fructose-6-phosphate amidotransferase (GFAT). Further, adenoviral mediated overexpression of GFAT increases levels of Id2. We conclude that hexosamine pathway mediate changes in Id2 level. Effect of glucose is cell type specific. While a similar effect is observed in Hepatocyte, little or no changes occur in L6 Myocytes and 3T3-L1 Adipocytes.

Id2 acts as negative regulator of transcription by forming inactive heterodimer with other members of the HLH family such as SREBP-1. Previously, work in the laboratory has shown that high levels of glucose prime J774.2 macrophages in such a way that insulin and leptin are able to reduce expression of hormone sensitive lipase (HSL). Here we observe that activity of mouse HSL promotor is increased when co-expressed with SREBP-1. The SREBP-1 induced increase in HSL promoter activity is attenuated upon co-expression with Id2, indicating that increased Id2 levels can mimic the effects of high glucose.

Id2 expression in J774.2 cells is affected by intracellular cAMP raising agents and insulin. Effect of cAMP is mediated by Epac through PI3 kinase dependent pathway. GSK3 is the end effector of both cAMP and insulin effect, its inactivation leads to up regulation of Id2 protein level.

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Abbreviations

Ab	antibody
ACAT	acylCoA cholesteryl acyl transferase
ApoB	apolipoprotein B
BSA	bovine serum albumin
bHLH	basic-Helix-Loop-Helix
cAMP	cyclic adenosine monophosphate
CE	cholesterol ester
DMSO	dimethylsulfoxide
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
ERK	extra cellular signal regulated kinase
FC	free cholesterol
FFA	free fatty acid
GPCR	G protein coupled receptor
HDL	high density lipoprotein
IRS	insulin receptor substrate
JAK-2	janus activated kinase-2
JNK	jun N-terminal kinase
LDL	low density lipoprotein
LPA	lysophosphatidic acid
LPL	lipoprotein lipase
MAPK	mitogen activated protein kinase
MCP	monocyte chemotacticprotein
MCS-F	macrophages colony stimulating factor
nCEH	neutral cholesterol ester hydrolase
OB-R	leptin receptor
Ox-LDL	oxidised LDL
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	plymerase Chain Reaction
PDE3B	phosphodiesterase 3B
PI3K	phosphoinositide 3-kinase
PIP3	phosphatidylinositol-3,4,5-phosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospho lipase C
PPAR	peroxisome proliferators activated receptor
SDS	sodium Dodecyl Sulfate
STAT	sognal transducers and activators of transcription
TAG	triglyceride
TNFα	tumout necrosis factor α
TZD	thiazolidinedione
VLDL	very low density lipoprotein

Chapter 1 Introduction

This thesis focuses on identifying mechanisms that might be involved in causing the increased rates of coronary artery disease in (people with diabetes mellitus). This chapter will therefore introduce atherosclerosis and diabetes and the mechanisms currently known to contribute to the development of these diseases.

1.1 Atherosclerosis

1.1.1 General characterisation

Cardiovascular disease is one of the major health problems worldwide. Atherosclerosis is the single most important contributor to the growing burden of the disease. Manifestations of atherosclerosis start in infancy and progress without exhibiting any symptom through out adult life. Later in life, advanced lesions are the underlying cause of clinical manifestations such as coronary artery disease, stroke, transient ischaemic attack and peripheral artery disease.

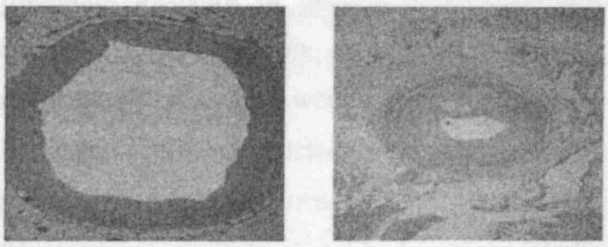


Figure1.1 Normal artery and artery affected by atherosclerosis with severe narrowing of the lumen

Atherosclerosis is characterised by an accumulation of lipid and fibrous elements in intima of medium and large arteries. Development of atherosclerotic lesions begins with endothelial dysfunction. Subendothelial LDL and oxidised LDL first infiltrate the intima and accumulate in the arterial wall, causing diffuse intimal thickening. Destabilisation of local homeostasis initiates an inflammatory response that leads to the complex recruitment and interaction of various immune cells. This progresses to fatty streak lesion with macrophages transforming into foam cells after uptake of large amounts of lipid. Fatty streaks are the earliest visible signs of atherosclerosis. In humans, fatty streak lesions can usually be found in the aorta in the first decade of life, the coronary artery in the second decade and the cerebral arteries in the third or fourth decades. Fatty streaks are clinically benign but form a precursor to more advanced atherosclerotic plaques with necrotic glue-like lipid material and cholesterol crystals in the central aromatous core covered with a fibrous cap structure. Later, plaques

may develop into advanced complicated lesions which can lead to ulceration, thrombosis and calcification.

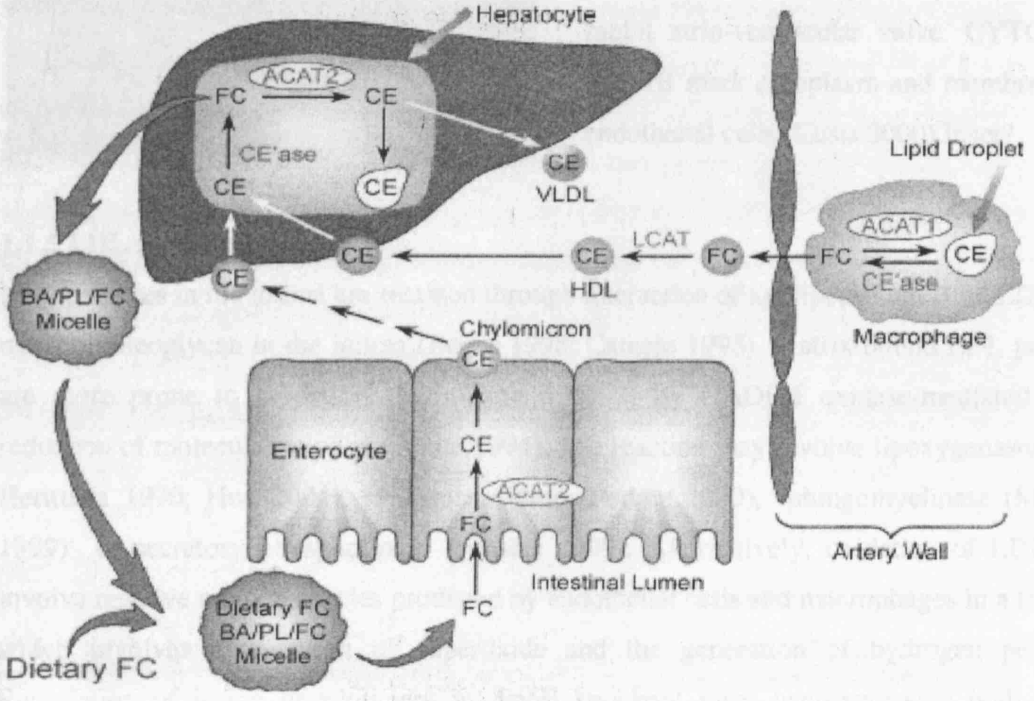
Hypercholesterolaemia is the main initiator of atherosclerosis. Clinical trials with Statins, (HMGCoA reductase inhibitors that block cholesterol synthesis), have shown that correcting hypercholesterolaemia leads to very significant reduction in mortality from coronary heart disease in both men and women regardless of the initial LDL cholesterol level(MRC/BHF 2002). In fact, hypercholesterolaemia is both necessary and sufficient for disease progression. Young children with familial hypercholesterolaemia develop coronary heart disease as early as 5 years of age without the presence of other risk factors (Brown 1986). In experimental mouse models such as the apolipoprotein E deficient mouse, the proatherogenic effects of low HDL levels only become apparent when LDL cholesterol level is higher than average (Rubin 1991; Li 1993). Thus, hypercholesterolaemia is the most vital part in atherosclerosis development and other risk factors must be considered as contributing factors to the process initiated by hypercholesterolaemia.

If hypercholesterolaemia is the main initiator of atherosclerosis, inflammation is the important mediator of disease progression from lesion initiation, lesion progression to thrombotic complication. A post-mortem study showed that arterial inflammation and macrophage infiltration were enhanced in patients with cardiovascular events (Fleiner 2004). Real-time PCR conducted in lesions of ApoE-/- mice showed that expression of proinflammatory cytokines, chemokines and their receptors increased in parallel with lesion development, cholesterol and body weight (Veillard 2004) while the removal of TNF-α from ApoE mice was atheroprotective (Branen 2004). Moreover, PPAR-γ agonists , a well established anti-inflammatory drug, reduced atherosclerosis through the reduction of VCAM-1, tissue factor expression and matrix metalloproteinase expression (Marx 1999; Marx 2001; Neve 2001). It also reduced thrombosis through a reduction in plasminogen activator inhibitor (PAI-1) and fibrinogen concentration, thus improving fibrinolysis (Kato 2000). In addition to cholesterol-lowering, statins have also been found to exert anti - atherogenic activity through anti-inflammatory action. They reduced leukocyte adhesion, antagonize macrophage activation, metalloproteinase production, and tissue factor procoagulant gene expression.

Since hypercholesterolaemia and inflammation are both important mediators of atherosclerosis, further studies must consider both responses to injury and elevated lipids together as equal partners in pathological development. Below, the mechanisms of cholesterol transport and lesion development are considered in detail.

1.1.2 Cholesterol Transport

Cholesterol in peripheral tissues is derived from dietary cholesterol absorbed by the intestine and cholesterol synthesised in the liver. Cholesterol ester is transported to peripheral tissue as a non-polar core component of lipoprotein, surrounded by polar phospholipids and apolipoproteins. According to composition and size, lipoprotein particles can be classified as chylomicrons, High Density Lipoprotein (HDL), Very Low Density Lipoprotein (VLDL), Intermediate Density Lipoprotein(IDL) and Low Density Lipoprotein (LDL). VLDL, IDL and LDL are the group of particles that deliver cholesterol from the liver to peripheral tissues. In the plasma lipoprotein particles encounter lipoprotein lipase (LPL) enzyme anchored along the surface of cells (Brunzell 1995). The triglyceride moieties in VLDL and IDL are hydrolysed by LPL, eventually turning these triglyceride-rich lipoproteins into denser lipoprotein particles such as LDL, which are absorbed by peripheral cells through receptor-mediated uptake. Peripheral cells obtain most of their cholesterol through this mechanism.



1.1.3 Endothelial dysfunction

The first event in fatty streak formation is endothelial dysfunction, a systemic and reversible disorder (Behrendt 2002) characterised by a reduction in bioavailability of vasodilators such as Nitric Oxide (NO). This is caused by increased production of reactive oxygen species

(Tomasian 2000), vasoconstrictor serotonin (Golino 1991) and endothelin-1 (Yang 1990). Together these changes lead to stronger vasoconstrictor response. Dysfunction of the endothelium promotes cellular permeability, lipoprotein oxidation, inflammation, smooth muscle cell proliferation, extracellular matrix deposition and platelet aggregation (Callow 2002).

1.1.4 LDL retention

Following endothelial dysfunction, LDL starts to accumulate in the sub-endothelial matrix. Because of differences in blood flow dynamics, there are preferred sites of lesion initiation within the arteries. Endothelial cells in tubular region of the arteries where lamina flow occurs are ellipsoid in shape and are aligned in the direction of flow. Cells around region of arterial branching or curvature, where flow is disturbed, have polygonal shapes and no particular orientation. Areas with more polygonal cells have increased permeability to macromolecules such as LDL and show more lesion development (Gimbrone 1999).

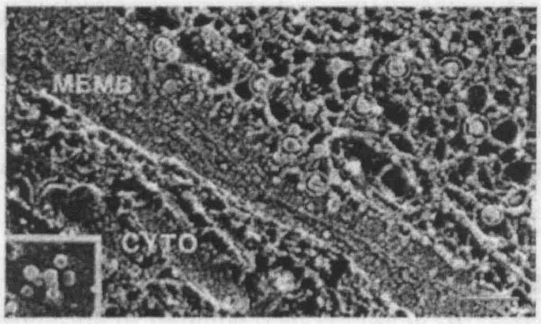


Figure 1.3 LDL retention Freeze-etched electron micrograph showing retention of 23nM particles of LDL in the matrix of rabbit atrio-ventricular valve. CYTO and MEMB mark cytoplasm and membrane of endothelial cells (Lusis 2000).Inset?

1.1.5 LDL modification

LDL particles in the intima are retained through interaction of apolipoprotein B on LDL with matrix proteoglycan in the intima (Boren 1998; Camejo 1998). Matrix-bound LDL particles are more prone to oxidation (Hurt-Camejo 1992) by NADPH oxidase-mediated direct reduction of molecular oxygen (White 1994). The reaction may involve lipoxygenases (Yla-Herttuala 1990; Huo 2004), myeloperoxidase (Podrez 2000), sphingomyelinase (Marathe 1999) or secretory phospholipase (Ivandic 1999). Alternatively, oxidation of LDL may involve reactive oxygen species produced by endothelial cells and macrophages in a reaction which involves dismutation of superoxide and the generation of hydrogen peroxide. Retardation of lesion development in ApoE knockout mice overexpressing both Cu/Zn superoxide dismutase and catalase and not the former alone showed that hydrogen peroxide but not superoxide contributes to the formation of oxidized lipids in atherosclerotic lesion (Yang 2004). Recently, *in vitro* analysis of atherosclerotic plaque revealed that ozone generated in lesion area through antibody-catalysed water oxidation pathway also contributes to the oxidative process. Cholesterol oxidation products generated from the reaction between

cholesterol and ozone subsequently turned macrophages into foam cells upon co-administration of LDL (Wentworth 2003).

1.1.6 Immune cells adhesion and transmigration

Under ordinary circumstances, the endothelial layer in contact with flowing blood resists firm adhesion of leukocytes. Soon after the initiation of atherogenic diets in rabbits, however, light microscopy revealed that the system undergoes inflammatory activation and blood leukocytes started to attach themselves to the endothelial cells that line the innermost layer of the artery (Poole 1958). This pathological recruitment and adherence of immune cells came about as a result of LDL accumulation.

Experiments involving apolipoprotein E deficient mice have shown that hypercholesterolaemia and LDL retention leads to pathological up regulation of VCAM 1 and ICAM-1, the key adhesion molecules for monocytes and T cells, on atherosclerotic prone sites where VCAM-1 is not expressed in normal mice (Nakashima 1998). Their importance has been confirmed in animal knock out model where removal of ICAM-1, VCAM-1 and P-Selectin, E-Selectin led to reduction in lesion size following lipid feeding (Cybulsky 1998; Dong 1998; Bourdillon 2000; Dong 2000).

Following the up regulation of adhesion molecules, adhesion of immune cells occurs in two stages. First, P and E selectins mediate rolling of monocytes and leukocytes on endothelial surfaces (Dong, Chapman et al. 1998). The following firm adhesion is then mediated by interaction between immune cell integrins and ICAM-1/ VCAM-1 on endothelial cells (Shih 1998). Firm adhesion of monocytes requires activation of integrin on immune cells by agonist-induced activation of G protein coupled cytokine receptor (Campbell, Hedrick et al. 1998). Monocytes express CC chemokine receptor 2, which requires monocyte chemoattractant protein -1 (MCP-1) for full activation (Han, Chen et al. 2003). This strong chemotactic factor is provided by endothelial cells and smooth muscle cells in the artery wall in response to ox-LDL (Han 1999). Subsequent to integrin activation, firm adhesion occurs and transmigration through the endothelial layer ensues. Ox-LDL itself has a strong chemotactic property for monocytes and this accumulation in the subendothelial layer contributes to monocytic cells

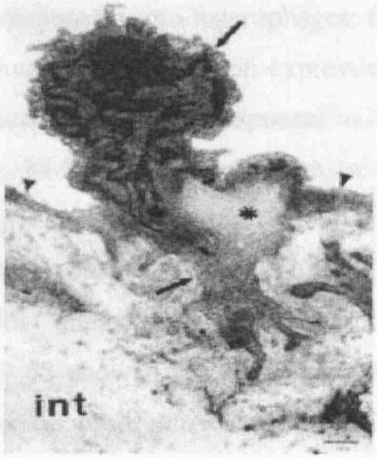


Figure 1.4 Macrophage transmigrating across endothelial layer

recruitment (Cushing 1990). Transmigration is shown in figure 1.4. The cell marked with an arrow is a monocyte.

In the subendothelial space, immune cells are exposed to high concentrations of oxidised LDL (Cushing 1990) and angiotensin (Nobuhiko 2004). This stimulates the production of (MCP-1) from immune cells, leading to a positive cycle of immune cell recruitment and chemoattractant production. Mice lacking either MCP-1 or its receptor CCR2 develop significantly reduced atherosclerotic lesions, pointing to a key role of MCP-1 and CCR2 in lesion development (Boring 1998; Gu 1998).

1.1.7 M-CSF promotes monocyte differentiation into macrophages

In the subendothelial space, monocytes undergo differentiation into macrophages through M-CSF mediated mechanism (Takahashi 2001). M-CSF is produced by infiltrating T lymphocytes and macrophages (Clinton 1992; Ruan 1995). Its expression is up regulated in response to ox-LDL and it is overexpressed in both animal and human models of atherosclerosis (Clinton 1992; Rosenfield 1992). The importance of M-CSF in fatty streak development has been confirmed when ApoE or LDLR and M-CSF double knock out mice developed smaller lesion than ordinary ApoE or LDLR knock out mice (Qiao 1997).

M-CSF activates the following steps in the conversion of monocytes into macrophages: the expression of Scavenger Receptor Type A expression, the expression of CD36 expression, and the production of cytokines and growth factor (De Villiers 1994). These responses to M-CSF are further potentiated by oxLDL (Hamilton 1999). M-CSF also contributes to an increase in the number of macrophages in the lesion area by serving as a strong survival, co-mitogenic and activation stimulus for macrophages *in loco (in situ?)* (Takahashi 2001; Cai 2004). Studies involving double immunostaining with monoclonal antibodies for proliferating cell nuclear antigen and for macrophage CD 68 showed that approximately 1.95% of macrophages in fatty streak lesions actually contain proliferative capacity. The proliferative capacity increased during fatty streak lesion and declined in atherosclerotic plaques (Rosenfeld 1990).

Macrophages in the subendothelial space produce many proinflammatory cytokines such as interleukin (IL)1, IL-6, IL-8, IL-10, IL-12 and tumour necrosis factor (TNF)-α in response to ox-LDL(Liu 1997; Huber 1999; Pinderski Oslund 1999; Ross 1999; Eihage 2001). This leads to heightened activation of circulating blood monocytes as well as increased monocytic recruitment and transmigration (Heidenreich 1999). Overall, there is progressive recruitment

and transmigration of monocytes and T lymphocytes but not neutrophils to the sites of developing lesions.

1.1.8 Uptake of modified LDL by macrophages in the subendothelial layer

After undergoing differentiation, macrophages take up modified LDL retained in the subendothelial space. It remains ambiguous whether this process contributes to overall removal of cholesterol from the lesion or facilitates formation of fatty streak (Kruth 2004). Nonetheless, the uptake mechanism has been under extensive analysis.

Evidence suggests that modified LDL, such as oxidised LDL or aggregate LDL, are the principle particles taken up by macrophages during foam cell formation (Goldstein 1979). In support of this theory, murine monoclonal antibodies against oxidised phosphatidylcholine of oxLDL demonstrated intracellular accumulation of oxLDL in macrophages and macrophage-derived foam cells in human atherosclerotic lesions (Itabe 1994). Incubation of inactivated macrophages with native LDL even at very high concentrations does not lead to foam cell formation because the LDL receptors are downregulated by a negative feedback control through a common mechanism that exists in fibroblast and lymphoid cells. In contrast to native LDL, ox-LDL does not bind to the LDL receptor. Oxidative modification of LDL causes fatty acid fragmentation that creates highly reactive intermediates such as aldehydes and ketones, which then complex with ApoB, increasing the net negative charge of LDL by abolishing the positive charge of ε amino group of lysine. As lysine residues are important for interaction between LDL and LDL receptor, charge modification prohibits LDL particles from interacting with the LDL receptor (Mahley 1983). Extensive degradation of the polyunsaturated fatty acid in the sn-2 position of phospholipids by oxidation (Boullier 2000) allows LDL to interact with scavenger receptor type A (Kodama 1988), CD 36 receptor (Endemann 1993) or Lectin-like oxidized LDL receptor (LOX-1) instead. Expression of scavenger receptors is not under a negative feedback mechanism; hence uptake of ox-LDL occurs according to external ox-LDL concentration regardless of intracellular cholesterol level. Expression level of scavenger receptors increases with proinflammatory cytokines such as tumour necrosis factor -α and interferon-γ. The level of scavenger receptor type A also increases during lesion progression, reaching its peak at the stage of fatty streak lesion while the level of CD36 is more marked in macrophage foam cells rather than in macrophages (Naito 1992). The contribution of uncontrolled uptake of ox-LDL by scavenger receptors to lesion development has been confirmed by work in knock out mice and in Japanese autopsy cases. ApoE/CD36, ApoE/ scavenger receptor type A or LDLR/ scavenger receptor type A double knockout mice showed reduction in lesion size (Suzuki 1997; Sakaguchi 1998; Fabbriao 2000), while atherosclerotic lesions were absent in

an 80 year old Japanese man with type I CD 36 deficiency. Recently stem cell transplantation experiments in mice showed that ApoE knockout mice with macrophages specific CD36 deficiency were profoundly protected against atherosclerosis with an 88.1% reduction of lesion area throughout the aortic tree (Febbraio 2004).

Recently, Kruth et al showed that native LDL may contribute directly to foam cell formation in activated macrophages, although this was not the case in inactivated cells. Series of experiments in PMA-activated human monocyte derived macrophages showed that activated macrophages took up native LDL through fluid phase macropinocytosis, a receptor-independent mechanism. The process was dependent on actin, PKC, Rho-GTPase and phosphatidylinositol 3-kinase signalling. M-CSF as well as oxidised LDL, acetylated LDL and aggregated LDL were able to act as activators of macropinocytosis. Activated macrophages were able to take up substantial amount of native LDL even at very high native LDL concentration of around 0.5 to 2 mg/ml in carotid intima and atherosclerotic plaque through this process (Kruth 2004). Because activated macrophages are abundant in lesion areas, macropinocytosis could be a major contributor to foam cell formation.

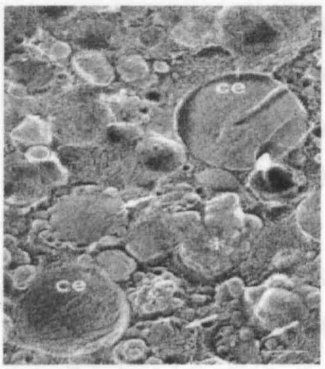


Figure 1.5 Cholesterol ester accumulation: Freeze etch electron micrograph of the cytoplasm of a macrophages foam cell in the intima of rabbit fed a high fat diet for 2 weeks. Large lipid droplets with the onion skin configuration are typical of cholesterol ester. * shows compartment with aggregate LDL.

1.1.9 Cholesterol uptake and break down

Using LDL that had been radiolabelled, either in its protein component or in its cholesteryl ester component, Goldstein et al showed that subsequent to the uptake, LDL is delivered to the lysosome as a unit. Both receptor-mediated and nonreceptor-mediated LDL enter the same ketaconazole (inhibitor of transport exiting lysosome) sensitive cholesterol trafficking pathway (Kruth 2004). In the acidic compartment, the protein component of the lipoprotein particle is hydrolysed and released from the cell while acidic cholesterol esterase catalyses the hydrolysis of cholesterol ester into free cholesterol. Subsequently, free cholesterol (FC) generated from the acidic compartment along with the FC component of lipoproteins are distributed to various cellular organelles. Most studies suggest that FC is first directed to the plasma membrane (Lange 1998) before further transport to the endoplasmic reticulum and

the mitochondria, where FC is converted to 27-hydroxycholesterol by the mitochondrial enzyme sterol 27-hydroxylase (Bjorkhem 1994).

Transport of FC to the endoplasmic reticulum contributes to cholesterol homeostasis through series of negative feedback pathways. One feedback pathway occurs when FC directly interacts with sterol-sensing domain of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate limiting enzyme in cholesterol biosynthesis, causing the enzyme to degrade (Roitelman 1992). In another pathway interaction of FC with the sterol-sensing domain of SCAP protein prevents proteolytic cleavage that, in the absence of sterol, leads to the activation of SREBP and further up regulation of cholesterolgenic genes (Brown 1997).

At the endoplasmic reticulum FC can be esterified into cholesterol ester to protect the cells against FC-mediated cytotoxicity. Accumulation of FC in the membrane is cytotoxic because imbalance of free cholesterol-phospholipid ratio in the plasma membrane restricts conformational freedom of integral membrane proteins and inhibits their proper functions (Yeagle 1991). FC crystallisation also damages cellular organelles (Kellner-Weibel 1999), eventually causing cytotoxicity. The accumulation of FC is known to provoke a number of metabolic responses in macrophages including increased phospholipid synthesis associated with post-translational up-regulation of CTP:phosphocholine cytidylyltransferase, increased expression of mRNA encoding for IL-8 and MCP-1 and FAS-dependent apoptosis (Yao 2000). As macrophages cannot break down FC, esterification process serves as a means to move free cholesterol out of the plasma membrane into the cytoplasm where it is stored as cholesterol ester, the most abundant form of lipid droplet in foam cells. In addition to cytoplasmic deposits, part of ox-LDL-derived cholesterol and cholesterol ester can form deposits in lysosomal compartment. This pool of sterol deposit in foam cell may prove particularly resistant to removal by efflux mechanism (Brown 2000).

1.1.10 Re-esterification of cholesterol : ACAT enzyme

The esterification process in macrophages is catalysed by Acyl coenzyme A:cholesterol acyltransferase 1 (ACAT1) in a process involving cholesterol and fatty acyl CoA (Chang 1997). The expression of ACAT1 increases as monocytes differentiate into macrophages. In these cells, intracellular FC is exposed to ACAT1 through an energy dependent process involving the actin cytoskeleton (Lange 1998). An increase in activity of ACAT following an increase in cellular FC occurs through an allosteric activation not an increase in ACAT expression (Cheng 1995; Chang 1997); hence in normal circumstances, the increase in activity of ACAT1 only occurs when FC levels reach a particular threshold level above the ambient cellular cholesterol concentration (Xu 1991).

On the other hand, ACAT activity and expression can increase independently of FC concentration under pathological conditions. Levels of ACAT increase post differentiation with interferon-γ and all-transretinoic acid through a pathway involving STAT1 (Yang 2001). It is also up regulated by TGF β-1, a common cytokine in atherosclerotic lesions (Hori 2004). The pathological increase in ACAT expression and activity by interferon-γ leads to overall reduction in HDL-mediated cholesterol efflux from macrophages (Panousis 2000). Insulin and leptin also increase ACAT1 activity regardless of FC concentration when J774.2 macrophages have been primed with high glucose overnight. This leads to accelerated CE accumulation (O'Rourke 2002).

In addition to a majority of ACAT found in the ER, a small portion of ACAT1 has been found at a perinuclear site near the trans-Golgi network and endocytic recycling compartment which are both cholesterol-rich membranes (Khelef 2000). Perhaps here ACAT may directly influence endocytic trafficking by regulating the FC content of these organelles.

It is still uncertain whether inhibition of ACAT1 will prove to be an attractive therapeutic strategy against atherosclerosis. While a few ACAT-specific inhibitors show promising results in animal or cell-line based experiments, as yet no compound has been shown to exhibit clear hypolipidaemic activity in a clinical trial. The ACAT inhibitor Avasimibe also caused mild increase in LDL cholesterol while failing to lower risk of coronary artery disease (Tardif 2004). Perhaps, this is due to a more complex role of ACAT in cholesterol homeostasis. Work by Fazio et al showed that deletion of ACAT1 gene from macrophages of LDLR deficient hyperlipidaemic mice actually increased lesion size (Fazio 2001). This is primarily caused by increased macrophage apoptosis due to cytotoxic effects from free cholesterol. Lipids from apoptotic macrophages augment plaque necrotic core while cellular debris increases inflammatory response, leading to an increase in the lesion area and an increase in free cholesterol accumulation in the arterial wall. Later, Dove et. al showed that ACAT1 deficiency led to a disruption of cellular free cholesterol efflux despite an upregulation of ABCA1, a mediator of cholesterol efflux. ACAT deficiency also led to a 27% increase in free cholesterol from acetylated LDL (Dove 2004). This evidence suggests that cholesterol ester formation is an important protection against cytotoxic effects of FC and that ACAT could contribute to cholesterol efflux through a more complex mechanism than previously believed. The attempt to reduce cholesterol ester formation by inhibiting ACAT may disrupt the intricate balance in an unfavorable manner.

1.1.11 Cholesterol ester hydrolysis : Hormone-sensitive lipase

Although initial transformation of FC to CE may protect cells from cytotoxic effects of FC, overaccumulation of CE is detrimental for the organism. For example, in macrophages, accumulation of CE leads to formation of foam cells and fatty streak lesions. Cells are protected from CE overaccumulation by cholesterol efflux. However, before FC can be exported from the cell, CE formed by ACAT has to be rehydrolysed to FC by a cytoplasmic neutral cholesterol ester hydrolase (nCEH). The hydrolysis and re-esterification occurs continuously in the cells, therefore CE overaccumulation, such as those in foam cells, usually occurs as a result of an imbalance in ACAT and nCEH activity.

The exact identity of neutral cholesterol ester hydrolase in macrophages remains uncertain. The finding that neutral cholesterol ester hydrolase activity in J774.2 and Raw macrophages can be nearly completely blocked with hormone sensitive lipase (HSL) specific antibody (Small 1989) first suggested that HSL is responsible for neutral cholesterol ester hydrolase activity in macrophages. Subsequent studies further confirmed the notion. First of all, HSL mRNA has been detected in humans and mouse macrophages (Khoo 1993; Reue 1997). (^{32}P)orthophosphate labelling studies coupled with immunoprecipitation showed that HSL in intact mouse peritoneal macrophages was phosphorylated following administration of dibutyryl cAMP and IBMX concomitantly with an increase in nCEH activity (Small 1991). Finally, Overexpression of HSL in THP-1 macrophages by adenovirus-mediated gene delivery removed cholesterol ester from cells preloaded with acetylated LDL completely and Raw 264.7 cells stably overexpressing HSL exhibited 2-3 fold increase in nCE activity when loaded with lipid (Escary 1998; Okazaki 2002).

Paradoxically, peritoneal macrophages from HSL knockout mice showed unchanged neutral cholesteryl ester hydrolysis despite the fact that they do in other tissues (Osuga 2000). There is a possibility that in its absence there is a compensatory increase in the expression of another enzyme capable of fulfilling neutral cholesterol esterase activity. Other than HSL, Human Cholesterol Ester Hydrolase similar to Rat Hepatic Neutral Cholesterol Ester Hydrolase cloned by Ghosh et al has been detected in THP-1 monocyte and macrophages as well as in human peripheral blood monocytes and macrophages. When overexpressed in agm (?) cells stably overexpressing ACAT, this esterase caused a reduction in total lipid droplet and intracellular CE content without increasing FC levels. However, it has not been proven as a sole provider of cholesterol esterase activity in mouse or human macrophages through immunoprecipitation. Alternatively, removal of HSL may affect another part of the cholesterol regulatory pathway such that total cholesterol remains constant. For example, removal of HSL from white adipose tissue reduced cellular triglyceride synthesis through a

reduction in activities of adipogenic enzymes and reduction in glucose uptake to such an extent that total triglyceride remained at the level of wild-type mice (Zimmermann, Haemmerle et al. 2003). Therefore, there is a possibility of a parallel reduction in other parts of cholesterol ester cycle in macrophages from HSL knockout mice.

In macrophages, nCEH activity determines the rate of cholesterol efflux. Work on atherosclerosis susceptible white carneau pigeons by Yancey and St Clair showed that a slow rate of cholesterol ester clearance was due partly to defect in cholesterol ester hydrolysis while work in THP-1 human monocyte derived macrophages cell line showed that the rate of free cholesterol efflux was determined by the activity of neutral cholesterol ester hydrolase (Brown 1980). Therefore, the activation of nCEH activity may prove to be a viable therapeutic strategy. However, when HSL was overexpressed in mouse macrophages without co-overexpression of cholesterol acceptor, a two-fold increase in lesion development occurred. The increase was revealed once ApoA1 was co-expressed (Choy 2003). Increased lesion development arises because FC generated by nCEH is a preferred substrate for ACAT (Klansek, Warner et al. 1996) and also acts as ACAT activator (Chang, Lee et al. 1998), hence FC generated is re-esterified immediately into CE by ACAT in a continuous cycle when efflux acceptors are limiting. For overexpression of HSL to be atheroprotective, efflux mechanisms must be upregulated simultaneously. Under physiological condition, HSL is maintained at low levels in macrophages. Nonetheless its activation could be beneficial when the subjects suffer from other pathological development.

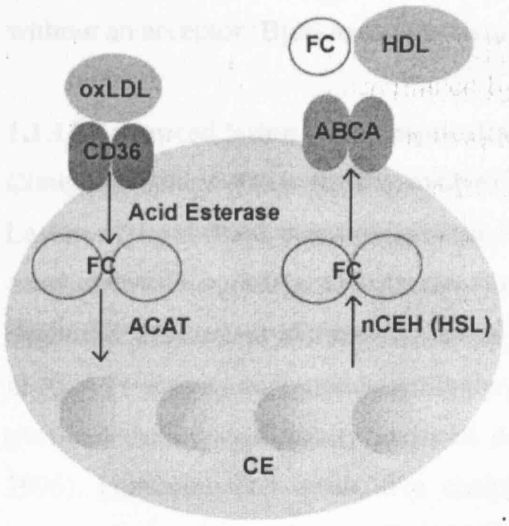


Figure 1.6 Cholesterol ester cycle in macrophages Ox-LDL particles are taken up by scavenger receptors. The cholesterol ester moiety is hydrolysed by acid cholesterol esterase, releasing FC. ACAT converts free cholesterol into cholesterol ester CE deposit while HSL converts CE into FC in a continuous cycle for ABCA-1 mediated efflux from the cells.

1.1.12 Cholesterol efflux

Cholesterol efflux is the main mechanism protecting macrophages from FC cytotoxicity. Macrophages can export their intracellular cholesterol to High Density Lipoprotein (HDL)

through an ApoE-dependent pathway. Severe atherosclerosis development in ApoE deficient humans, ApoE-deficient mice and macrophage-specific ApoE-deficient mice supports the notion that ApoE plays an important atheroprotective role (Fazio 1997).

Intracellular free cholesterol is exported through the cell membrane via ATP-binding cassette transporters-1 (ABC1) (Lawn 1999). Free cholesterol is then accepted on the membranes by apolipoprotein AI (ApoAI), a major protein component of HDL (Kellner-Weibel 2003). Transgenic mice over expressing ApoAI showed increase plasma HDL level and reduced susceptibility to diet-induced atherosclerosis (Rubin 1991) and overexpression of ApoAI in ApoE knockout mice reduced lesion size (Benoit 1999). PPAR-γ agonists also exert antiatherogenic effects through an enhancement in reversible cholesterol transport by up regulating ABCA-1 and ApoAI. In addition to a role in reverse cholesterol transport, HDL also inhibits lipoprotein oxidation. The antioxidant properties of HDL are due in part to serum paraoxonase, an esterase carried on HDL that can degrade biologically active oxidised phospholipids (Hegele 1999; Shih 2000).

Another pathway is the spontaneous elimination of cholesterol by the metabolism of cholesterol to 27-hydroxycholesterol by sterol 27-hydroxylase, a widely distributed mitochondrial cytochrome P450 enzyme which is expressed highly in human macrophages. The product, 27-hydroxycholesterol, is one of the most abundant oxysterosl in human atherosclerotic lesion and macrophage foam cells and is produced in culture in macrophages in response to cholesterol loading. The polar nature of the molecule allows it to be exported without an acceptor (Bjorkhem, Andersson et al. 1994).

1.1.13 Advanced lesion and complications

Clinically benign fatty streaks evolve into fibrotic lesions with clinical manifestations. Layers of lipid-filled macrophages usually intersperse with smooth muscle cells, T cells, connective tissue matrix of collagen fibril, elastic fibre and proteoglycan. During lesion evolution, FC content increases in lesion macrophages. This occurs as a result of a decrease in ACAT1 activity and a reduction in the expression of ABCA1 protein (Albrecht 2004). FC overload causes cytotoxicity and cell death through apoptosis and necrosis (Mitchinson 1996). Following cell death, free cholesterol and cholesterol ester are released into the growing mass of necrotic core (Ball 1995; Tabas 1996). Yet, the contribution of macrophage apoptosis to lesion development remains controversial. Recently Liu et al have shown that reduction in macrophage apoptosis by reconstitution of LDLR-/- mice with Bax -/- bone marrow actually increased atherosclerosis development. Macrophage apoptosis might serve as an atheroprotective effect in this circumstance (Liu 2004).

The complex web of cytokines produced by macrophages and T cells secreted through an inflammatory response also begins to recruit smooth muscle cells. Upon migration, vascular smooth muscle cells lost their contractile phenotype. They revert back to synthetic phenotype and start to secrete many growth factors and cytokines as well as reverting the mature $\alpha 1\beta 1$ integrin phenotype back to the $\alpha 2\beta 1$ phenotype, allowing it to respond further to other mitogenic and chemotactic signal. Elevated level of homocysteine appears to damage endothelium in the area around fatty streak lesion, stimulating the migration or proliferation of smooth muscle cells around the area (Gerhard 1999). The renin-angiotensin system mediates the effect of raised blood pressure on lesion development through a stimulation of smooth muscle cell growth and increases extracellular matrix production. Raised blood pressure also stimulates expression of platelet-derived growth factor, a potent mitogen for smooth muscle cells (Negoro 1995).

Traditionally, it has been accepted that the growth of benign atheroma occurs continuously in progressive fashion over time and there is a boundary between progression and complication phase of atherosclerosis. Nowadays clinical observations are challenging this view and the boundary between progression and complication stage of atherosclerosis has become blurred. A series of serial angiographic studies have shown that many coronary arterial lesions in humans developed stenoses discontinuously (Yokoya 1999). Current evidence obtained from microscopic pathoanatomy of plaques suggests that physical disruption of plaques may trigger thrombosis and thus promote sudden expansion of atheromatous lesions (Davies 1996). There are three possible causes of plaque disruption (Virmani 2002).

Firstly, a superficial erosion of endothelial cells covering the intima. Inflammation is the main underlying cause of endothelial dequamation. Production of local proinflammatory cytokines and attack by killer T cell lead to endothelial cell apoptosis, which in turn increases the recruitment of more immune cells to the lesion area. Inflammatory mediators and oxidised lipoprotein also stimulate the expression and activation of type 1 matrix metalloproteinases specialising in degradation of endothelial basement membrane (Rajavashisth 1999).

A second cause of sudden lesion progression is intraplaque haemorrhage or in situ thrombosis. Plaque develops fragile micro vessels as they evolve. These vessels are prone to rupture and deposit of fibrin and haemosiderin provide evidence for disruption of microvessels in atherosclerotic plaques (deBoer 1999). Thrombin generated from this process leads to the cleavage of fibrinogen and potent stimulation of smooth muscle cell

migration and proliferation. Thrombin also triggers platelet release of growth factor such as PDGF, causing accelerated proliferation of smooth muscle cells. Activated platelets also release TGF-ß, the most potent stimulus for interstitial collagen synthesis by smooth muscle cells (Libby 2002).

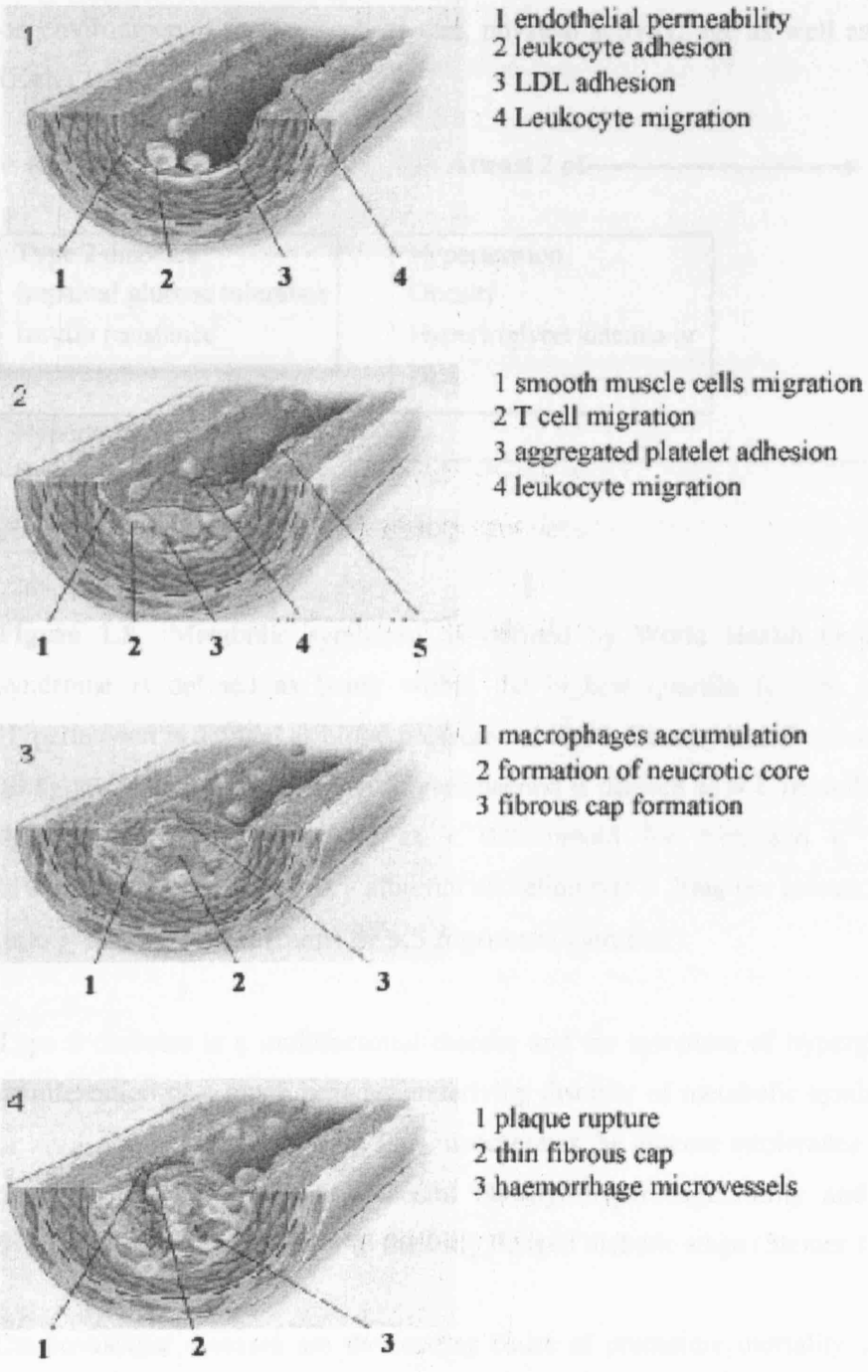
The final and most common mechanism of plaque disruption is the fracture of fibrous cap. Interstitial collagen molecules produced by smooth muscle cells confer most of the tensile strength on the fibrous cap (Lee 1997). Proinflammatory cytokines such as Interferon-γ can inhibit collagen production by these cells. Simultaneously other proinflammatory cytokines found in atheroma plaque such as IL-1β,TNF-α and CD40 ligand stimulate overexpression of many interstitial collagenases, stromolysin and gelatinases (MMP) in mononuclear phagocytes and endothelial and smooth muscle cells (Saren 1996). Mast cells in the lesion may also release TNF-α as well as serine proteinases that can activate latent MMP proenzymes (Kovanen 1995). The net result of reduction in collagen production and increased collagen degradation during inflammation renders fibrous cap susceptible to fracture under haemodynamic stresses.

Plaques tend to rupture around the shoulder where the cap is the thinnest. This area is most heavily infiltrated with immune cells and usually contains debris of dead macrophages (Van Der Wal 1994). Plaques with thick fibrous cap tend to be more stable while those with thin fibrous cap, large core of extracellular lipid, high density of macrophages containing lipid and reduced numbers of vascular smooth muscle cells tend to pose more serious risk of fissure. As far as risk assessment is concerned, plaque composition and vulnerability of plaque is a more important factor to consider rather than severity of stenosis.

Fissure of fibrous caps allows the coagulation factors in the blood stream to be in contact with tissue factor, a glycoprotein that is the primary inhibitor of blood coagulation, in the lipid rich core. Thrombus formation is initiated as platelets are activated by thrombin generated from the coagulation cascade and by contact with the intimal compartment. A balance of pro-coagulant pathway and fibrinolytic mechanisms determine whether occlusive and sustained blood clot or limited mural thrombus would form after plaque disruption. If coagulation mechanism outweighs the fibrinolytic effect, thrombus may block the vessel persistently and acute myocardial infarction can result. If fibrinolytic mechanism were more prominent, limited mural thrombus would form after plaque rupture. Healing follows in response to thrombin generated during blood coagulation. During healing of the ruptured plaque, activated platelet release PDGF and TGF-ß, causing smooth muscle cell proliferation and collagen accretion. Consequently, migration, proliferation and extracellular matrices

production by smooth muscle cells thickens fibrous cap, turning fatty streak into fibrous plaque. Evolving plaque expand into the intima, further occluding the lumen if maximum compensatory vascular enlargement has been reached (Glagov 1987). Significant encroachment may lead to clinical manifestation of angina pectoris, however a large proportion of plaque ruptures probably cause no clinical symptom (Libby 2002).

Figure 1.7 atherosclerosis lesion progression (Ross 1999)



1.2 Diabetes as risk factor for atherosclerosis

Changes in human lifestyle and behavior over the last century have resulted in a dramatic increase in the incidence of diabetes mellitus worldwide. A study by King et al. shows that the number of adults with diabetes in the world will rise from 135 million in 1995 to 300 million by the year 2025. There will be a 42% increase in developed countries and an astonishing 170% increase in the developing countries (King 1998). Type 2 diabetes mellitus is the most prevalent form, accounting for more than 90% of cases. Its development depends on environmental factors such as diet, physical activity, age as well as genetic disposition (Kahn 1996).

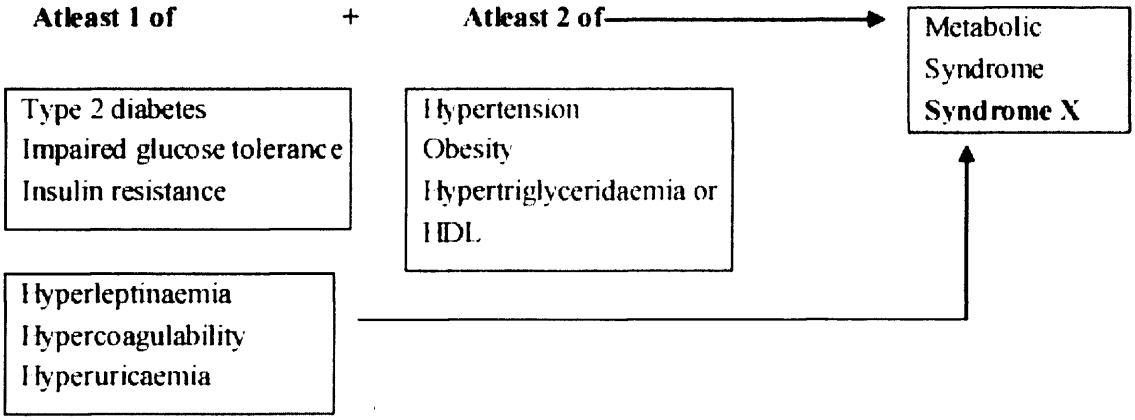


Figure 1.8 Metabolic syndrome as defined by World Health Organisation metabolic syndrome is defined as being within the highest quartile for the relevant population. Hypertension is defined as blood pressure > 140/90. Obesity is defined as body mass index > 30 kg per square meter. Hypertriglyceridaemia is defined as > 1.7mmol/l. Low high-density lipoprotein (HDL) is defined as < 0.9 mmol/l for men and < 1.0 mmol/lwomen. Microalbuminuria is a urinary albumin excretion rate > 20ug per minute or albumin/creatine ratio $\geq$ 2.5 mg/mmol (men) or 3.5 mg/mmol (women).

Type 2 diabetes is a multifactorial disease and the symptom of hyperglycaemia is often a manifestation of a much broader underlying disorder of metabolic syndrome (syndrome X) or insulin resistance syndrome. This incorporates the glucose intolerance, hyperinsulinaemia, dyslipidaemia, hypertension, visceral obesity, hypercoagulability and microalbuminuria. Syndrome X may occur prior to the fully fledged diabetic stage (Steiner 1994).

Cardiovascular diseases are the leading cause of premature mortality in diabetes patients. Numerous epidemiological studies have shown that the risk of cardiovascular disease

development for subjects with type 2 diabetes is higher compared with non-diabetic subjects. There is a decrease in the age of onset of cardiovascular disease and a 3-fold increase in incidence of asymptomatic ischaemia in subjects with diabetes compared to non-diabetic subjects (Kannel 1979; Stamler 1993; Stout 1993; Lee 2000; Khaw 2001; Bonora 2003; Hegazi 2003; Lee 2003; Otel 2003; McGuire 2004). This is accompanied by accelerated development of carotid atherosclerosis (Wagenknecht 2003). This excessive increase in risk cannot be fully accounted for by classical risk factors for cardiovascular disease development such as hyperlipidaemia and hypertension (Stamler 1993; Laakso 1998; Lehto 2000; Hegazi 2003). Evidence also shows that cardiovascular disease development in type 2 diabetes can occur prior to the onset of diabetes. It is therefore likely that atherosclerosis development is caused by a combination of risk factors that contribute to syndrome X which may precede the fully fledged diabetes stage. Together these risk factors are classified as non-traditional risk factors (Fonseca 2000; Saito 2000). In fact, incidences of coronary artery disease correlate very well with symptoms of the metabolic syndrome (Lamarche 1998; Bonora 2003).

With an expected increase in diabetes in the years to come, the burden on public health expenditure will be enormous. It is therefore vital to study the mechanisms leading to the cause of susceptibility to atherosclerosis, the main underlying cause of coronary artery disease, in order to prevent or ameliorate the condition. Both diabetes-derived non-traditional and traditional risk factors are discussed below.

1.2.1 Hypertension

Hypertension is a classical risk factor for cardiovascular disease. The incidence of peripheral vascular disease, aortic calcification, and coronary artery disease occurs more frequently when hypertension is present. Hypertension occurs more frequently in the diabetic population (Steiner 1994; Stern 1996). Hyperglycaemia and hyperinsulinaemia play an important role in hypertension since reduction in blood pressure has been observed in diabetic patient with better glycaemic control (Stern 1996). Hyperinsulinaemia enhances the expression of endothelin-1(Reaven 1996), a potent vasoconstrictor and causes a defect in several membrane ion transport system such as Na/K ATPase pump, Ca^{2+}-ATPase pump and the Na^+/H^+ antiporter system. This results in sodium retention and an increase in cytosolic calcium and growth of smooth muscle cells (Stern 1996).

1.2.2 Dyslipidaemia

Key components of diabetes dyslipidaemia are elevated serum VLDL-triglyceride, more abundant small and dense low density lipoprotein (sd-LDL) and decreased plasma HDL-C

level, especially HDL_2-C (Taskinen 1992; Brunzell 2002). High LDL cholesterol and low HDL cholesterol are widely recognised as cardiovascular risk factors (Howard, Cowan et al. 1998) and their correction through aggressive treatment with statin and fibrates successfully reduced cardiovascular disease incidence in the Diabetes Atherosclerosis Intervention Study (DAIS) (Investigators. 2001).

Insulin resistance is an important contributor to the elevation of VLDL level. Primarily, insulin resistance impairs normal suppression of fatty acid release from adipose tissue by insulin (Chen, Golay et al. 1987), even under hyperinsulinaemia. Also contributing to increased levels of fatty acid is insulin-stimulated increase in hepatic *de novo* fatty acid synthesis through SREBP-1c activation and subsequent upregulation of lipogenic enzymes such as fatty acid synthase and acetyl CoA carboxylase. In the mouse model of insulin resistance and hyperinsulinaemia, SREBP-1c expression is up regulated via IRS-1/PI3 kinase pathway (Matsumoto, Ogawa et al. 2002) despite down regulation of IRS-2 mRNA and insulin resistance (Shimomura, Matsuda et al. 2000). This increases hepatic *de novo* lipid synthesis in response to hyperinsulinaemia even under the general insulin resistance state. Consequently, fatty acid flux to the liver is elevated and overproduction of VLDL in the liver ensues (Malmstrom, Packard et al. 1997).

Normally insulin suppresses postprandial VLDL release from the liver when there is high level of chylomicrons transported from the intestine. In a healthy system, insulin's potentiation of ApoB degradation (Chirieac, Chirieac et al. 2000) and inhibition of microsomal transfer protein (MTP) expression (Lin, Gordon et al. 1995) inhibits hepatic VLDL particles assembly and secretion. Subsequent to the attenuation of these effects by insulin resistance such as those in obese diabetic mice (Bartels, Lauritsen et al. 2002) and sucrose-induced insulin resistant mice (Taghibiglou, Carpentier et al. 2000), the increase in MTP expression occurs in the liver. This leads to over production of VLDL even during the postprandial state when there is high level of intestinal chylomicrons. Overproduction of VLDL and competition with chylomicron saturates the removal pathway catalysed by endothelial lipoprotein lipase (Ginsberg and Illingworth 2001). In diabetic patients (Taskinen 1987), accompanying the increase in production of VLDL is a decrease in level of lipoprotein lipase. This shifts the lipid balance towards hypertriglyceridaemia.

Hypertriglyceridaemia and slower VLDL catabolic rate together lead to an increase in number of small and dense LDL particles. Small and dense LDL particles are well established as a cardiovascular risk factor (Carr MC 2001). In the Quebec Canadian Study, LDL particles with diameter less than 255 Å are associated with up to 4.5 fold increase in

risk of cardiovascular disease (St Pierre 2001). They are associated with higher plasma triglyceride concentration, lower HDL concentration as well as increased insulin resistance(Reaven 1993). Longer residence time of VLDL allows for increased transfer of cholesterol ester from LDL to triglyceride-rich lipoprotein and for triglyceride transfer from triglyceride rich lipoprotein to LDL, resulting in triglyceride-rich LDL particles. The enzyme hepatic lipase, which is up regulated in diabetes, then preferentially converts triglyceride rich LDL into small and dense LDL (Syvanne and Taskinen 1997).

Smaller LDL particles are more atherogenic because they have increased vascular permeability (Austin 1996) and increased affinity for the matrix, which leads to longer residence time in the intima (Wang, Greilberger et al. 2001). A study in vitro showed that they are more susceptible to oxidation and glycation (Tribble, Holl et al. 1992) and are hence are more likely to form atherogenic ox-LDL, which is taken up very readily by macrophage scavenger receptors (Austin 1996). Additionally ox-LDL promotes chemotaxis of monocytes into the vessel wall (Quinn 1987). It activates proinflammatory genes through NF-kB (Andalibi, Liao et al. 1993). The proinflammatory effect is clinically relevant since oxLDL is associated with increased level of inflammatory mediators such as C-reactive protein and TNF-α in the Atherosclerosis and Insulin Resistance Study (Hulthe and Fagerberg 2002).

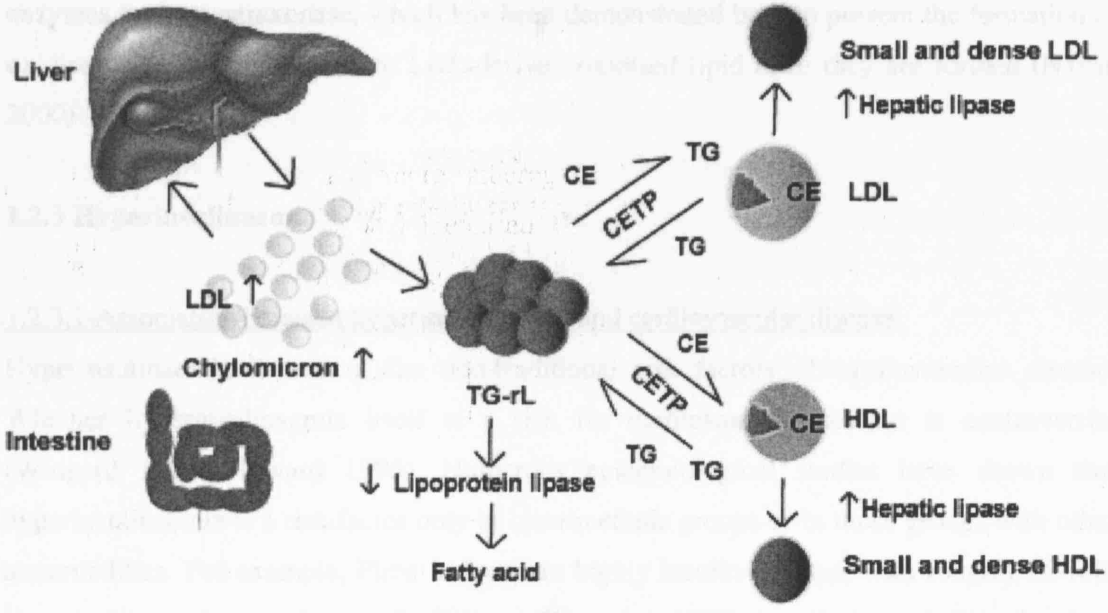


Figure 1.9 Insulin resistance as the cause of hypertriglyceridaemia (Syvanne and Taskinen 1997). Insulin resistance causes overproduction of VLDL from the liver, ultimately increasing TG-rL level. Reduction in LPL activity in diabetes increases residence time of TG-rL. This increases TG transfer to LDL and HDL, making them better substrate for hepatic lipase.

Previously , it was thought that hyperglycaemia and insulin resistance mediated a reduction in lipoprotein lipase activity (Yost, Froyd et al. 1995) and subsequent reduction in apolipoprotein remnant availability has been proposed as the central cause of the reduction in HDL level (Merkel, Eckel et al. 2002). However, kinetic studies have shown that patients with low HDL level tend to have high apoA catabolic rate but normal rate of ApoA production (Pont, Duvillard et al. 2002), hence this theory is unlikely to be very significant.

Another explanation arises from prolonged residence time in circulation of VLDL and the increase in hepatic lipase activity in diabetes (Syvanne, Ahola et al. 1995). The increased residence time increases cholesteryl ester transfer protein mediated exchange of cholesterol ester from HDL to VLDL and triglyceride from VLDL to HDL particles. The process enriches HDL particle core with triglyceride (Guerin, Le Goff et al. 2001). Since hepatic lipase preferentially hydrolyses triglyceride-rich HDL (Thuren, 2000), prolonged residence time of VLDL predisposes HDL to subsequent stages of catabolism (Lamarche, Uffelman et al. 1999). Together, increased triglyceride content and accelerated hydrolysis enhances clearance rate of HDL (Rashid, Barrett et al. 2002). A stable isotope study has confirmed that increased HDL catabolic rates do occur in patients with type 2 diabetes (Frenais, Ouguerram et al. 1997). Consequently, reduction in number of HSL particles reduces total cholesterol efflux from peripheral tissues and reduces antioxidant effects from HDL-bound enzymes such as paraxonase, which has been demonstrated both to prevent the formation of oxidised LDL and to inactivate LDL-derived oxidised lipid once they are formed (Navab 2000)

1.2.3 Hyperinsulinaemia

1.2.3.1 Association between hyperinsulinaemia and cardiovascular disease

Hyperinsulinaemia is one of the non-traditional risk factors of cardiovascular disease. Whether hyperinsulinaemia itself is a risk for cardiovascular disease is controversial (Wingard 1995; Howard 1996). Numerous epidemiological studies have shown that hyperinsulinaemia is a risk factor only in certain ethnic groups or in those groups with other abnormalities. For example, Pima Indians are highly insulin resistant with roughly 20-fold increased prevalence of type 2 diabetes (Knowler 1978) but the population develops relatively lower levels of coronary artery disease compared with Caucasians (Nelson 1990). This observation has been attributed to the lower LDL cholesterol levels in Pimas (Nelson 1990). Three large studies demonstrated that insulin levels correlate with coronary disease in multivariate analyses (Pyorala 1979; Welborn 1979; Ducimetiere 1980), but a review by Wingard et al showed that subsequent study in a smaller number of subjects showed no

association (Wingard 1995). Another study showed that correlation existed in men after adjusting for systolic blood pressure, use of medication, family history, triglyceride, apolipoprotein B, high LDL, low HDL cholesterol concentration (Despres 1996). However, in the Multiple Risk Factor Intervention Trial (MRFIT), hyperinsulinaemia is a risk factor only in men with apolipoprotein E 3/2 phenotype instead of the more common E 3/3 phenotype(Orchard 1994). In women, little relation has been identified between hyperinsulinaemia and heart disease, except in the Atherosclerosis Risk in Communities Study (Nabulsi 1995). A more recent study by Lehto et al., however, supports the notion that endogenous hyperinsulinaemia increases the risk of cardiovascular disease death in type 2 diabetes patients (Lehto 2000).

1.2.3.2 Insulin signalling cascade

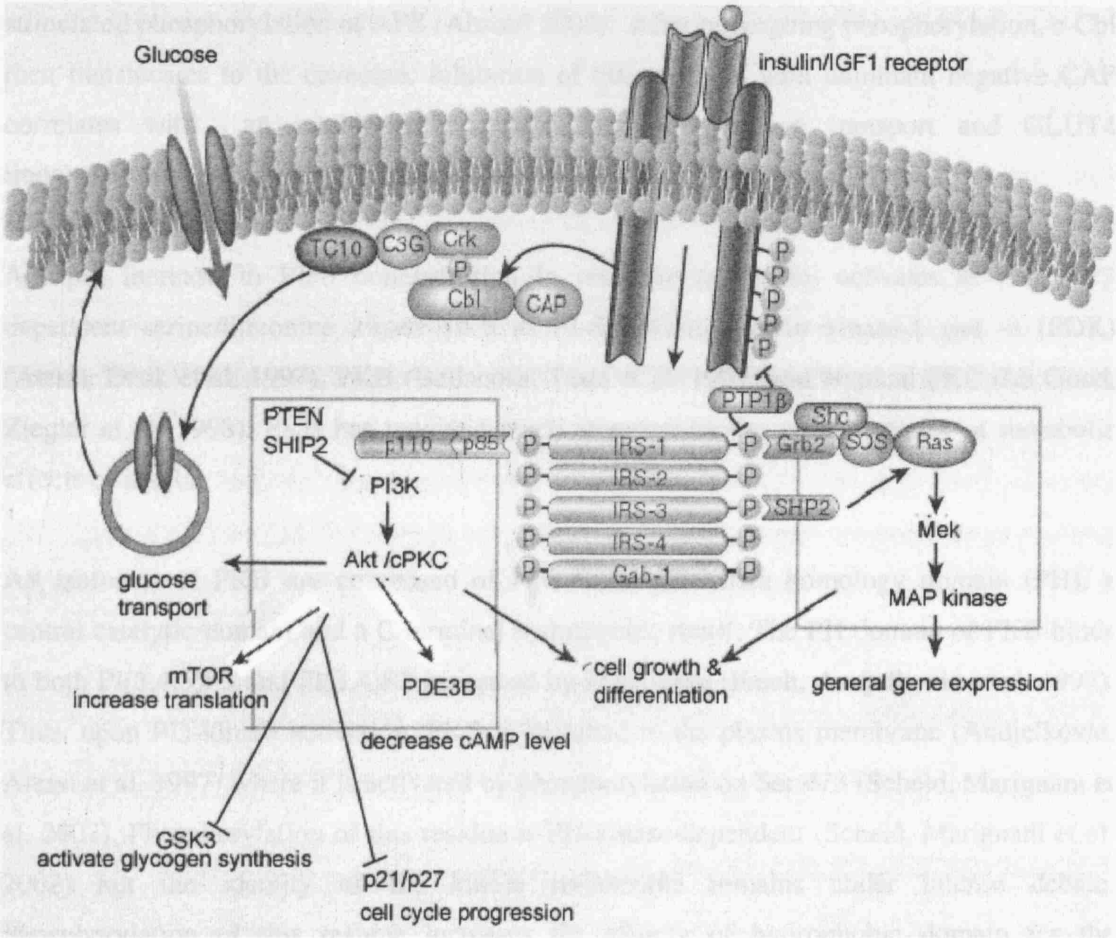
Insulin is secreted by pancreatic ß cells in response to increased plasma glucose concentration. It causes an increase in glucose uptake into fat and muscle tissue, a reduction in hepatic gluconeogenesis, an increase in glucose utilisation and storage as glycogen and triglyceride and reduction in feeding.

The insulin receptor is a heterodimeric membrane glycoprotein composed of two alpha and two beta subunits linked together by disulfide bonds. Binding of insulin to the alpha subunits causes conformational change, enabling binding of ATP to the beta subunit. This activates receptor autophosphorylation (Hubbard, Wei et al. 1994; Hubbard 1997), which in turn activates the receptor's kinase activity toward intracellular protein substrates. The main sites of autophosphorylation are Tyr 1158, 1160 and 1162 in the activation loop of the catalytic domain and Tyr 1328, 1334 in the carboxy terminal domain. Phosphorylation in the activation loop is essential for the receptor's kinase activity and the carboxy terminal phosphorylation sites are important for the receptor's mitogenic activity while juxtamembrane sites are important for the stability of receptor/substrate complex (Tavare and Siddle 1993).

The most well established downstream substrates of insulin receptor are the Insulin Receptor Substrate (IRS) family (White, Maron et al. 1985). Insulin receptor phosphorylates IRS proteins on the conserved YxxM motifs. Phosphorylation of the tyrosine residue increases IRS affinity towards other signalling molecules (White 1998). Knockout studies in mice showed that IRS-1 is vital for mitogenic effect of insulin while IRS-2 is important for metabolic regulation in response to insulin. IRS-1 knock out led to slight insulin resistance and severe growth retardation (Tamemoto, Kadowaki et al. 1994) while IRS-2 knock out led to severe insulin resistance (Withers, Gutierrez et al. 1998). Interestingly, ablation of several

alleles of IRS proteins (IRS-1 -/-IRS-2+/-) does not produce as severe insulin resistance phenotype as insulin receptor knock out, while IRS-1/IRS-2 double knock out mice are embryonic lethal (Withers, Burks et al. 1999). The phenotype is more severe than that of the insulin receptor and IGF receptor knockout mice (Louvi, Accili et al. 1997). These observations suggest that IRSs are not the only mediator of metabolic effects of insulin and that IRS plays additional role to mediate actions of other receptors.

Figure 1.10 Insulin signalling cascade (modify from Satiel and Kahn)



One of the main mediators of insulin effects downstream of IRS protein is the PI3 kinase enzyme. Blocking of its activity with wortmannin inhibits insulin-stimulated glucose uptake (Shimizu and Shimazu 1994), glycogen synthesis (Sakaue, Hara et al. 1995), lipid synthesis (Okada, Kawano et al. 1994) and downregulation of PEPCK expression (Sutherland, O'Brien et al. 1995). PI3 kinase catalyses the additions of phosphate on the D3 position of the inositol ring of phosphoinositol. The form of the enzyme activated by IRS proteins consists of regulatory subunits and catalytic 110kDa subunit. The regulatory subunits exist in many isoforms for example, p85 alpha, p85 beta, p55, p55 PIK. Lipid kinase activity of PI3 kinase

generates 3-phosphorylated inositides intracellular messenger (PIP3), which causes the recruitment and activation of several downstream effectors.

It has been reported that Insulin receptor also signals through PI3-kinase-independent pathways through c-Cbl. C-Cbl is a substrate of insulin receptor kinase in differentiated adipocytes not preadipocytes (Ribon and Saltiel 1997). The differences occur because of differential expression of the APS adapter protein which recruits Cbl to the insulin receptor (Ahn 2003). The interaction between APS with c-Cbl in adipocytes is dependent on insulin stimulated phosphorylation of APS (Ahmed 2000). After undergoing phosphorylation, c-Cbl then translocates to the caveolae. Inhibition of this pathway with dominant negative CAP correlates with an inhibition of insulin-stimulated glucose transport and GLUT4 translocation in a wortmannin-independent manner (Pessin and Saltiel 2000).

A rapid increase in PIP3 concentration in response to insulin activates several PIP3 dependent serine/threonine kinase such as PI-dependent protein kinase-1 and -2 (PDK) (Alessi, Deak et al. 1997), PKB (Bellacosa, Testa et al. 1991) and atypical PKC (Le Good, Ziegler et al. 1998). PKB has received much attention as the main mediator of metabolic effects of insulin .

All isoforms of PKB are composed of N-terminal pleckstrin homology domain (PH), a central catalytic domain and a C terminal hydrophobic motif. The PH domain of PKB binds to both PI(3,4,5)P3 and PI(3,4)P2 generated by PI3-kinase (Frech, Andjelkovic et al. 1997). Thus, upon PI3-kinase activation, PKB is recruited to the plasma membrane (Andjelkovic, Alessi et al. 1997) where it is activated by phosphorylation on Ser 473 (Scheid, Marignani et al. 2002). Phosphorylation of this residue is PI3-kinase-dependent (Scheid, Marignani et al. 2002) but the identity of the kinase responsible remains under intense debate. Phosphorylation of this residue increases the affinity of hydrophobic domain for the hydrophobic and phosphate binding pockets created by a cleft formed at the junction of the alpha, beta-helix, alpha C-helix and beta5-sheet in the N-lobe of the kinase domain. The binding leads to increased stability of the catalytic core, increasing phospho-group transfer rate by 10-fold (Frodin, Antal et al. 2002; Yang, Cron et al. 2002). A second regulatory phosphorylation on PKB, which is dependent on primary phosphorylation on Ser-473 (Scheid, Marignani et al. 2002), occurs on Thr308 residue in the activation T loop. Thr-308 is phosphorylated by PDK-1 (Alessi, James et al. 1997; Stephens, Anderson et al. 1998). Gene targeting in mouse embryonic stem cells showed that deletion of PDK-1 resulted in inhibition of PKB and complete inhibition of Thr308 phosphorylation (Williams, Arthur et al. 2000), further confirming the role of PDK-1 in PKB phosphorylation. PKB and PDK-1 are

brought into close proximity through the interaction between PKB's hydrophobic domain and PDK1 (Frodin, Antal et al. 2002) as well as the inclusion of both proteins into lipid micelle surface through interaction with PI(3,4,5,)P3 or PI(3,4)P2 (Alessi, James et al. 1997). Phosphorylation on Thr308 is essential for PKB activity as demonstrated by lack of PKB activity in alanine mutants (Alessi, Andjelkovic et al. 1996).

Upon activation, PKB can translocate to the nucleus (Andjelkovic, Alessi et al. 1997) and phosphorylate substrates with RxRxxS/T consensus (Obata, Yaffe et al. 2000). The first substrate identified for PKB is GSK3. GSK3α and GSK3β are phosphorylated on Ser 21 and 9 respectively by PKB. The phosphorylation results in inactivation of GSK3 (van Weeren, de Bruyn et al. 1998). Unphosphorylated GSK3 is active and phosphorylates and inactivates glycogen synthase (Imazu, Strickland et al. 1984). In response to insulin stimulation, GSK3 is phosphorylated and inactivated, leading to activation of glycogen synthase and increased glycogen synthesis (Cohen, Nimmo et al. 1978). PKB activation also links insulin-signalling cascade to cAMP signalling cascade. By phosphorylating and activating PDE3B in adipocytes (Kitamura, Kitamura et al. 1999), PKB allows insulin to lower intracellular cAMP level in a tissue specific manner. PKB also mediates the effect of insulin on mRNA translation by phosphorylating and activating mTOR (Nave, Ouwens et al. 1999). Besides the role in metabolism, PKB contributes to the promotion of cell cycle progression. PKB phosphorylates p21 (Zhou, Liao et al. 2001), and P27 (Viglietto, Motti et al. 2002) inhibiting their potential to arrest the cell cycle.

The insulin signalling cascade is terminated by protein tyrosine phosphatases (PTPs) catalysed dephosphorylation of tyrosyl-phosphorylates proteins (Tonks and Neel 2001). PTP1B member of the family was first proposed as a negative regulator of insulin signalling through its ability to inhibit insulin stimulated GLUT4 translocation (Chen, Wertheimer et al. 1997) and its ability to bind directly to activated insulin receptors (Seely, Staubs et al. 1996). To date, many studies have confirmed this notion. Mice lacking PTP1B exhibited enhanced insulin sensitivity and increased insulin receptor phosphorylation in liver and muscle (Elchebly, Payette et al. 1999). Overexpression of PTP1B in various culture models decreased insulin receptor and IRS-1 tyrosyl phosphorylation and IRS-1 associated PI3 kinase activity (Venable, Frevert et al. 2000,Kenner et al., 1996), while PTP1B silencing through RNAi in hepatoma cell line increased insulin signalling (Xu, Li et al. 2005).

Mechanistic studies involving BRET have shown that PTP1B interacts with insulin receptor in the endoplasmic reticulum even under basal conditions, possibly prohibiting autophosphorylation. This interaction is strengthened once the receptor is activated (Boute,

Boubekeur et al. 2003). PTP1B specifically dephosphorylates tyrosine 1150, 1151 and 960 on beta subunit of insulin receptor (Galic, Hauser et al. 2005), leading to inhibition of PI3 kinase and PKB phosphorylation. Another phosphatase that recognises insulin receptor as substrate is TC-PTP (Galic, Klingler-Hoffmann et al. 2003). Insulin signalling and PKB phosphorylation are prolonged in mouse embryo fibroblast (Galic, Klingler-Hoffmann et al. 2003) and HepG2 cells (Meng, Buckley et al. 2004) lacking this protein. The activity of TCPTP does not overlap with those of PTP1B. It specifically dephosphorylates tyrosine 972 instead of residues targeted by PTP1B (Galic, Hauser et al. 2005).

While PTP1B and TCPTP act on insulin receptors, the lipid phosphatase PTEN attenuates insulin signalling by dephosphorylating PI(3,4,5)P3 at the 3' phosphate, (Maehama and Dixon 1998) leading to decreased levels of this phospholipid and reduces PKB activity (Cantley and Neel 1999). The importance of PTEN as inhibitor of insulin signalling has been demonstrated by hyperactivity of PI3 kinase/PKB pathway (Hyun, Yam et al. 2000) and hypersensitivity to insulin in mammalian system with PTEN deletion (Stiles, Wang et al. 2004). In addition to PTEN, SHIP2 is another lipid phosphatase that inhibits PI3 kinase activity. It dephosphorylates the 5' phosphate of the inositol ring from PI(3,4,5)P3. Overexpression of SHIP inactivates insulin signalling and inhibits downstream metabolic effects of insulin by blocking PKB activation (Sasaoka, Hori et al. 2001; Wada, Sasaoka et al. 2001; Sasaoka, Wada et al. 2004). Work carried out in adipocytes suggests that PTEN and SHIP are non-redundant and that inhibitory effects are tissue-specific. For example, depletion of PTEN by RNAi in 3T3-L1 markedly enhanced insulin-stimulated PKB and GSK3 phosphorylation as well as glucose uptake while removal of SHIP has no effect on the same process (Tang, Powelka et al. 2005). The tissue-specific nature of lipid phosphatases is further strengthened after the discovery of 5'-phosphatase named skeletal muscle and kidney-enriched inositol phosphatase (SKIP) (Ijuin, Mochizuki et al. 2000; Ijuin and Takenawa 2003). This phosphatase performs a similar function to PTEN in heart, skeletal muscle and kidney. Interference with SKIP increases PKB phosphorylation and overexpression of SKIP inhibits GLUT4 translocation, glucose uptake and glycogen synthesis in L6 myocytes.

Inhibition of negative regulators of insulin resistance may be a good therapeutic strategy. Normalised plasma glucose, reduced hyperinsulinaemia, lowered levels of gluconeogenic enzyme expression and increased insulin sensitivity have been observed in diabetic mice after they were treated with antisense to PTP1B in liver and fat (Zinker, Rondinone et al. 2002; Gum, Gaede et al. 2003). Reduction in the levels of lipogenic enzymes has also been observed in fat tissue of the same system (Rondinone, Trevillyan et al. 2002) while PTP1B knockout mice display enhanced whole body glucose disposal, increased insulin-invoked

suppression of hepatic glucose production, decreased adiposity and resistance to high fat diet-induced obesity (Elchebly, Payette et al. 1999; Klaman, Boss et al. 2000). A similarly beneficial effect has also been observed after inhibition of lipid phosphatases. Specific inhibition of PTP1B in mouse liver through hydrodynamic injection of RNAi increases insulin signalling (Xu, Li et al. 2005) while liver-specific deletion of PTEN results in increased insulin sensitivity in the liver and improved overall glucose tolerance (Stiles, Wang et al. 2004) and muscle-specific deletion of PTEN causes enhanced insulin sensitivity in soleus muscle and protection from high fat diet-induced obesity (Wijesekara, Konrad et al. 2005).

1.2.3.3 Insulin resistance

Insulin resistance syndrome is one of the hallmarks of type 2 diabetes. Impaired glucose uptake and impaired inhibition of hepatic glucose production then results from insulin resistance (Kahn 1998). Initially the body compensates for decreased insulin sensitivity of target tissues by increasing insulin secretion. This leads to a condition known as hyperinsulinaemia, which may be present in a substantial proportion of the normal "non-diabetic" population (Reaven 1996). The most widely accepted cause of impaired insulin signalling is excess circulating fatty acid in some individual. Intravenous fat infusion (Yu, Chen et al. 2002), high fat feeding (Wang, Obici et al. 2001) and obesity (Houmard, Tanner et al. 2002) have been shown to attenuate PI3-kinase activation. Intravenous fat infusion study showed that the inhibition is caused by DAG-mediated activation of PKC and subsequent serine/threonine phosphorylation of IRS-1 on ser 307 (Yu, Chen et al. 2002). Accumulation of triglyceride may come about as a result of leptin resistance since leptin limits fatty acid accumulation in non-adipose tissues through activation of fatty acid oxidation in healthy animals (Shimabukuro, Koyama et al. 1997). In support of the role of leptin in triglyceride accumulation and insulin resistance, administration of leptin to diet-induced obese mouse reduces adipose mass and intramuscular triglyceride content by 40% and 60% respectively and this is associated with reversal of muscle insulin resistance (Buettner, Newgard et al. 2000).

The pathological hyperleptinaemia that accompanies obesity also inhibits insulin actions. In adipocytes, leptin directly reduces the maximal amount of bound insulin (Walder, Filippis et al. 1997). It impairs insulin stimulated glucose transport, glycogen synthase and lipogenesis in a dose dependent manner. At 2nM insulin sensitivity was reduced, while 30nM leptin abolishes the effects of insulin completely (Muller, Ertl et al. 1997). Impairment of insulin stimulated MAPK activity, glycogen synthase kinase phosphorylation and insulin receptor tyrosine phosphorylation also occur in rat adipocytes when a high amount of leptin is

administered centrally over 7 days (Perez, Fernandez-Galaz et al. 2004). Inhibitory effects have also been observed in other cell types. In L6 myotubes, pre-incubation with leptin attenuates insulin-stimulated glucose uptake through p38MAPK inhibition although there is no effect on PI3 kinase, IRS or PKB phosphorylation (Sweeney, Keen et al. 2001). Finally, exposure of HepG2 cells to very high concentration of leptin of 900ng/ml attenuates several insulin-induced activities including IRS-1 tyrosine phosphorylation and gluconeogenesis (Cohen, Novick et al. 1996).

1.2.3.4 Mechanisms linking hyperinsulinaemia to atherosclerosis

The effects of hyperinsulinaemia on various cells types in atherosclerotic lesion have been studied but insulin seems to contribute to both vascular protection and vascular injury. Vascular protective effects of insulin are derived from the stimulation of endothelial nitric oxide (NO) production (Wu 1994). Many vascular protective effects of NO arise from stabilisation of IkB and subsequent reduction in NF-kB action (Peng 1995). Consequently, NO inhibits vascular muscle cell growth (Garg 1989) and intimal hyperplasia (Davies 1994). NO also inhibits the expression of adhesion molecule such as vascular cell adhesion molecule-1, E-selectin and intercellular adhesion molecule 1(De Caterina 1995). Importantly NO inhibits the production of pro-inflammatory cytokine such as TNF-α and the production of proatherogenic chemokines such as monocyte chemoattractant protein-1 (MCP-1)(Zeiher 1995). In summation, NO reduces binding of inflammatory cells such as macrophages and monocytes to vascular wall and reduces the thrombotic process by preventing platelet aggregation and adhesion to vascular wall(Loscalzo 1992).

On the contrary, insulin has many potentially proatherogenic effects. Insulin has been found to potentiate the effect of platelet-derived growth factor on VSMC proliferation and migration (Banskota 1989) through MAPK-dependent pathways (Xi 1997). Insulin also promotes the expression of plasminogen activator inhibitor-1 (PAI-1), a physiological inhibitor of fibrinolysis, in VSMC (Stolar 1988). The observation has been replicated *in vivo* in rabbits (Nordt, Sawa et al. 1995). An increase in PAI-1 levels leads to fibrin deposition along coronary arteries (Stout 1993). This stimulates cell proliferation and vascular wall thickening (DeYoung, Tom et al. 2001), a determinant of atherosclerosis development, and stimulates accumulation of low density lipoprotein (Juhan-Vague 1991).

Insulin also affects many aspects of macrophage function. Insulin binds to and is internalised by macrophages in a manner similar to other insulin target tissues such as hepatocytes. However, a difference between macrophages and other insulin-sensitive tissues lies in the GLUT isoforms expressed in response to insulin: macrophages express GLUT 1

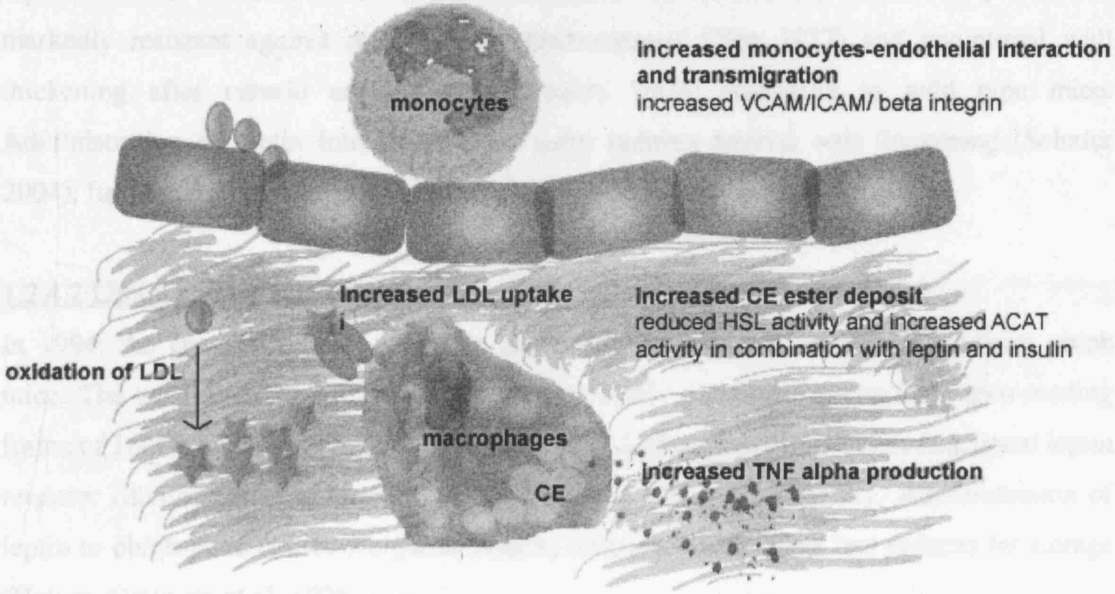
and GLUT 3 transporters in response to insulin instead of GLUT4 (Daneman 1992)(Estrada, 1994). Despite these differences, a study carried out in monocytes showed that monocytic glucose uptake increases in response to insulin (Daneman 1992). Conditions found in diabetes also affect the macrophage response to insulin. For example, monocytes obtained from patients with insulin resistance or diabetes bind less insulin due to decreased concentration of insulin receptors (DeFronzo 1979). Hyperinsulinaemia also increases LDL binding and intracellular cholesterol synthesis in macrophages (Stout 1990). At normal fasting blood glucose concentration, insulin decreases cholesterol ester accumulation in J774.2 cells. However at high glucose concentrations, insulin causes a marked increase in cholesterol ester and triglyceride accumulation which is associated with a reduction in hormone sensitive lipase activity and expression and an increase in ACAT activity (O'Rourke 2002).

In addition to the direct effects on lipid accumulation, insulin also affects the release of proinflammatory cytokine release from arterial cells. A study has shown that it increases the expression and the secretion of tumour necrosis factor-α (TNF-α) from THP-1 cells in a dose-dependent manner (Iida 2001). Consequently, proinflammatory cytokines such as TNF-α induce expression of chemotactic proteins and growth factors such as matrix metalloproteinases, vascular cell adhesion molecule-1 and heparin binding epidermal growth factor-like growth factor in vascular endothelial and smooth muscle cells (Yoshizumi 1992; Taherzadeh, Fen et al. 1993; Galis 1995; Spiecker 1997). These latter cytokines promote migration and adhesion of monocytes to endothelial cells (Libby 1995). Insulin also directly promotes endothelial cell-monocyte adhesion. Treatment of cultured human endothelial cells with insulin increases VCAM-1 expression via p38 MAPK dependent pathway and this process leads to increased adhesion of U937 monocytes to vascular endothelial cells (Madonna, Pandolfi et al. 2004).

The ultimate effects of insulin will depend on the balance of the vaso- protective and vaso-injury effects. In obese, insulin resistant and diabetic patients, disturbance in the PI3K signalling cascade has been observed, causing a reduction in glucose uptake into fat and muscle cells as well as a reduction in NO production (Laakso 1990; O'Brien 1997). On the other hand, the MAPK cascade appears to function normally in an animal model of insulin resistance (Jiang 1997). Hence, the vascular damage effect of insulin is maintained while the vascular protective effect is impaired in insulin resistance and diabetic states. Since many of the pathological effects are evident in macrophages, a main contributor to lesion formation, it is important to analyse other macrophage intracellular machinery affected by hyperinsulinaemia in order to identify novel targets for intervention. Thus far Troglitazone,

an insulin-sensitizing agent, has been shown to reduce VSMC growth by reducing Elk-1 phosphorylation, thereby reducing activation of c-fos (Law 1996), and reducing macrophage proinflammatory cytokine production (Jiang 1998). By analysing the effect of hyperinsulinaemia on macrophage gene expression, a new target for intervention could be identified.

Figure 1.11 Proatherogenic effect of insulin on macrophages. Insulin increases the interaction between macrophages and endothelial cells. It increases binding of LDL to scavenger receptor , cholesterol ester accumulation and secretion of proatherogenic cytokine such as TNF -α.



1.2.4 Hyperleptinaemia and cardiovascular disease

1.2.4.1 Association between hyperleptinaemia and cardiovascular disease

Dyslipidaemia is a major link between obesity and atherosclerosis, especially in relation to increases in VLDL secretion from the liver, hypertriglyceridaemia and an increase in small dense LDL and a reduction in HDL cholesterol (Klannemark 1998). However, analysis of the West of Scotland Coronary Prevention Study has shown that hyperleptinaemia is an independent risk factor for cardiovascular disease in its own right independent of body-mass-index (Wallace 2001). The result is supported by a case control study from Northern Europe that reports plasma leptin concentration as a risk factor for myocardial infarction and hemorrhagic stroke (Soderberg 1999; Soderberg 1999). Elevated serum leptin level is detected in patients with advanced chronic heart failure (Leyva 1998; Schulze 2003). Moreover, human plasma leptin concentration is independently associated with the intima-media thickness of the common carotid artery, an early atherosclerosis marker (Ciccone

2001), as well as myocardial wall thickness in hypertensive insulin-resistant men (Paolisso 1999). In patients with angiographically confirmed atherosclerosis, plasma leptin level is a predictor of future cardiovascular events independent of lipid status (Wolk 2004). Human leptin level also correlates well with other markers of metabolic syndrome such as plasma triglyceride, apolipoprotein B level, systolic blood pressure (Haffner 1999). BMI-adjusted hyperleptinaemia correlates well with higher C-reactive protein, hyperinsulinaemia and hypercholesterolaemia (Buettner, Bollheimer et al. 2002).

Similar observations have been made in murine models ob/ob mice which lack functional leptin exhibit fewer atherosclerotic lesions than other obese rodents (Nishina 1994). They are markedly resistant against diet-induced atherosclerosis (Yen 1977) and neointimal wall thickening after carotid artery injury (Bodary 2002) compared to wild type mice. Administration of leptin into these ob/ob mice induces arterial wall thickening (Schafer 2004), further confirming the link between leptin and cardiovascular disease.

1.2.4.2 Leptin and intracellular signalling

In 1994, the gene encoding leptin was discovered through positional cloning from ob/ob mice. The gene encodes a 4.5 kb mRNA and a highly conserved amino acid open reading frame of 16kDa (Zhang, Proenca et al. 1994). Mice lacking leptin (ob/ob) or functional leptin receptor (db/db) are characterized by hyperphagia and morbid obesity. Administration of leptin to ob/ob mice causes weight reduction, decreases food intake and reduces fat storage (Halaas, Gajiwala et al. 1995).

Adipose tissue is the main site of leptin synthesis although it is produced at lower level in skeletal muscle and placenta (Zhang, Proenca et al. 1994). Plasma leptin level changes in accordance with energy intake. The level increases after refeeding (Weigle, Duell et al. 1997) or overfeeding (Kolaczynski, Ohannesian et al. 1996) and decreases after fasting (Boden, Chen et al. 1996). A number of studies have shown that these changes occur as a result of insulin and glucose-mediated changes in leptin secretion. Insulin increases leptin gene expression *in vivo* and *in vitro* (Havel 2000) and insulin infusion in humans increases circulating leptin concentration (Utriainen, Malmstrom et al. 1996). Mechanistic *in vitro* studies conducted in isolated adipocytes suggest that insulin-mediated glucose metabolism not insulin per se stimulates leptin production. For example, when glucose transport or glycolysis was blocked, insulin action on leptin expression was inhibited despite high concentration of insulins (Mueller, Gregoire et al. 1998).

The first receptor specific for leptin was isolated from mouse carotid plexus by expression cloning (Tartaglia, Dembski et al. 1995). Subsequent analysis uncovered six splice variants of the receptor, encoded as variants a-f (Tartaglia 1997). Leptin receptor has the characteristics of type I cytokine receptors, containing homologous domain in the extracellular region, two di-sulfide link in the N terminus, and a WSXWS motif in the C terminus. Isoforms of leptin receptor share the same extracellular domain but differ in the length of the intracellular domain. Only the long form of leptin receptor (Ob-Rb) contains the box 2 motif necessary for activation of JAK/STAT pathway (Tartaglia 1997). Details concerning signalling capacity of the short form are starting to emerge, but despite exhibited signalling capacity, short forms of leptin receptors alone are not sufficient to mediate leptin actions on energy homeostasis or neuroendocrine control. Homozygous mouse lacking Ob-Rb but possessing a full complement of short leptin receptor isoforms still develop hyperphagia, cold intolerance, obesity, insulin resistance and obesity(Kowalski, Liu et al. 2001).

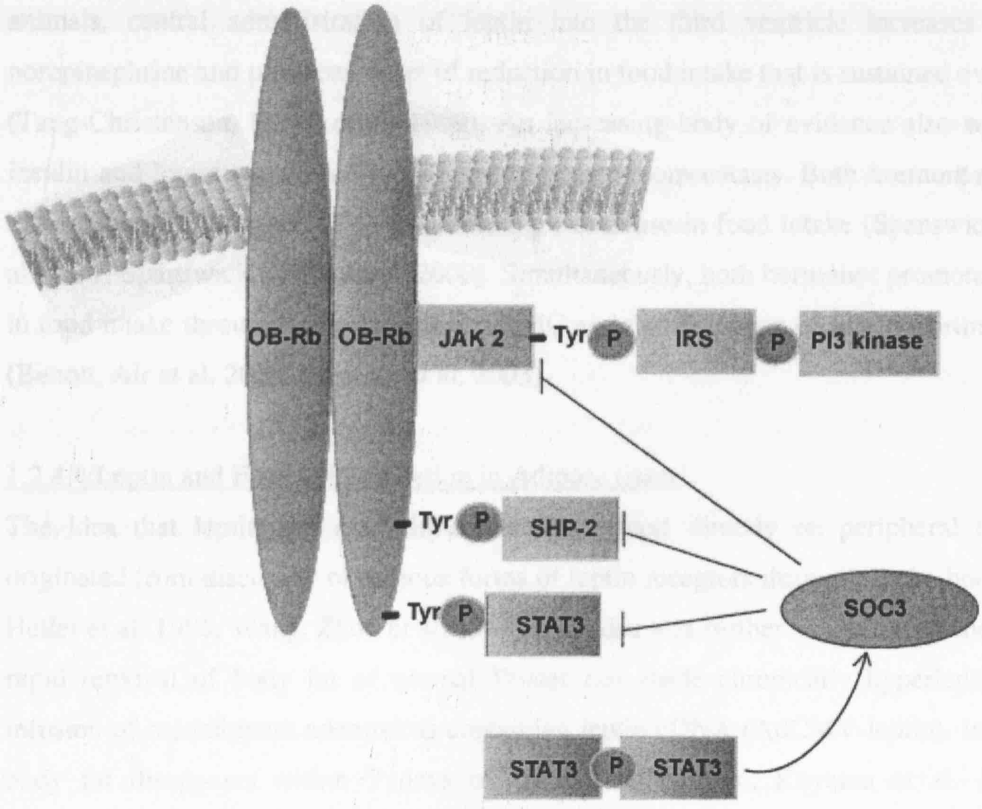
Figure 1.12 Obese (ob/ob) mouse compare with lean control (http://newsfromrussia.com/images/newsline/0-88-leptin-mouse.jpg)

In common with other cytokine receptors, the leptin receptor does not contain intrinsic enzymatic activity. Instead, it relies on non-covalently associated tyrosine kinases of the JAK family. Unliganded leptin receptor exists as a homodimer. Upon ligand binding, conformational changes occur enabling transphosphorylation and activation by JAK2, which constitutively associate with box 1 and box 2 of leptin receptor (Bahrenberg, Behrmann et al. 2002). Activated JAK2 then phosphorylates other tyrosine residues within the JAK2/receptor complex, enabling downstream signalling (Banks, Davis et al. 2000). STAT3 is recruited to Y1138 on the receptor via SH2 domains (Bates, Stearns et al. 2003). Tyrosine phosphorylated STAT3 then undergoes homodimerisation and nuclear translocation (Muraoka, Xu et al. 2003). The role of STAT3 in the central leptin anorectic signalling has been established clearly by a study of rodents with a homologous replacement of LRb by a receptor mutant for Tyr1138. There is dysregulation of POMC and AgRP expression in the hypothalamus and the control of feeding in these rodents (Bates, Stearns et al. 2003). Phosphorylation of Tyr 985 of leptin receptor creates a binding site for the carboxyterminal

SH2 domain of SHP-2, leading to activation of Erk signalling (Bjorbaek, Buchholz et al. 2001).

JAK2 tyrosine phosphorylation also mediates signalling independently of tyrosine phosphorylation of leptin receptor. This pathway leads to activation of IRS-2 and PI3 kinase, enabling a cross talk between insulin and leptin signalling cascade in C2C12 muscle cells. (Kellerer, Koch et al. 1997). Indirect evidence for the involvement of this pathway in central leptin actions came from the observation that IRS 2 (-/-) mice displayed increased feeding and decreased metabolic rate in the presence of increased leptin (Burks, de Mora et al. 2000). Subsequently leptin has been found to stimulate PI3-kinase in the hypothalamus and pharmacological blockade of PI3-kinase activity blocked the anorectic effect of leptin *in vivo* (Niswender, Morton et al. 2001). Although the exact mechanism leading to activation of PI3-kinase has not been solved, it seems to have very important physiological role.

Figure 1.13 leptin signalling cascade



After prolonged stimulation, the leptin receptor reaches a refractory state, effectively limiting efficacy of high concentration of leptin during chronic exposure (Dunn, Bjornholm et al. 2005). This is caused by induction of Suppressor of cytokine signalling 3 (SOCS3) by leptin (Emilsson, Arch et al. 1999). SOCS3 is up regulated through JAK2-STAT3 pathway. When expressed, SOCS3 binds to Tyr 985 of the receptor to mediate inhibition of STAT3

signalling (Bjorbak, Lavery et al. 2000). The feedback inhibition is abolished following mutation of Tyr1138 and by RNAi inhibition of SOCS3 expression (Dunn, Bjornholm et al. 2005). Downstream from the receptor, protein tyrosine phosphatase-1B (PTP-1B) recognises a motif on JAK-2 and reduces leptin signalling. Deletion of PTP-1B enhances leptin sensitivity and STAT3 phosphorylation (Zabolotny, Bence-Hanulec et al. 2002).

1.2.4.3 Leptin and central control of energy balance

The regulation of food intake by leptin has been attributed to the effects of leptin on the central nervous system, particularly in the basomedial hypothalamus, a site of high OB-Rb expression (Elmquist, Bjorbaek et al. 1998). Here, leptin acts on arcuate anabolic neurons, which synthesises neuropeptide Y (NPY) and agouti related protein (AgPR), as well as on arcuate catabolic neurons, which produce propiomelanocortin (POMC). The former neuron decreases food intake when activated while the latter decrease food intake when activated. The effect of centrally administered leptin on food intake and weight loss has been demonstrated clearly in rodents and rhesus monkeys (Caro, Sinha et al. 1996). In these animals, central administration of leptin into the third ventricle increases circulating norepinephrine and produces onset of reduction in food intake that is sustained over 24 hours (Tang-Christensen, Havel et al. 1999). An increasing body of evidence also suggests that insulin and leptin coordinate the control of energy homeostasis. Both hormones inhibit the neuropeptide Y producing neurons, causing a decrease in food intake (Spanswick, Smith et al. 1997; Spanswick, Smith et al. 2000). Simultaneously, both hormones promote a decrease in food intake through the synthesis of POMC and the activation of melanocortin C receptor (Benoit, Air et al. 2002; Choi, Li et al. 2003).

1.2.4.4 Leptin and Fatty acid oxidation in Adipose tissue

The idea that leptin can exert lipid-reducing effects directly on peripheral tissues first originated from discovery of various forms of leptin receptors throughout the body (Kieffer, Heller et al. 1996; Wang, Zhou et al. 1996). The idea was further supported by the finding of rapid removal of body fat of normal Wistar rats made chronically hyperleptinaemic by infusion of recombinant adenovirus containing leptin cDNA (AdCMV-leptin). In these rats, body fat disappears within 7 days of the infusion (Chen, Koyama et al. 1996). The magnitude of response raises the possibility of a direct hormone to cell action of leptin in addition to the central response. Subsequent studies in vivo then showed that leptin acted directly on adipocytes to reduce total triglyceride accumulation without changing total free fatty acid level in blood plasma (Shimabukuro, Koyama et al. 1997).

One of the explanations for the lack of changes in total free fatty acid level is an increase in adipocyte fatty acid oxidation. Leptin up regulates carnitine palmitoyl transferase-1 (CPT-1), an enzyme responsible for the transport of long chain fatty acid such as oleic acid across mitochondrial membrane for fatty acid oxidation and Krebs cycle (Greville and Tubbs 1968), and acyl CoA oxidase after 3 hours incubation in adipose tissue of lean rats (Zhou, Shimabukuro et al. 1997; Wang, Lee et al. 1999; Ceddia, William et al. 2000). Leptin also indirectly activates CPT by inhibiting the production of malonyl CoA by ACC. Because CPT activity is allosterically downregulated by malonyl CoA (McGarry 1998), inhibition of FAS by leptin would indirectly lead to activation of CPT. Moreover, leptin also activates enzymes along the Krebs cycle in adipocytes. Together with an increase in CPT, this upregulation further increases the overall fatty acid oxidation in response to leptin (Ceddia, William et al. 1999).

An alternative explanation for the lack of changes in total plasma free fatty acid is the induction of fatty acid efflux from adipocytes to other tissues with high fatty acid oxidative capacity (William, Ceddia et al. 2002). The second theory is supported by the fact that induction of oxidation only occurs to exogenously derived fatty acid, not intracellular fatty acid, in isolated rat adipocytes. By increasing fatty acid export to other tissues with higher oxidation rates than mitochondria-poor adipocytes, overall plasma fatty acid level may appear constant *in vivo* because leptin increases fatty acid oxidation rate in other tissue in similar proportion to the release from adipose tissue.

In addition to an increase in fatty acid oxidation, leptin also reduces *de novo* fatty acid synthesis through reduction in expression of fatty acid synthase in preadipocytes (Bai, Zhang et al. 1996), and reduction in expression of fatty acid synthase as well as acetyl CoA carboxylase enzymes in adipose tissue from lean rats (Wang, Lee et al. 1999; Zhou, Wang et al. 1999).

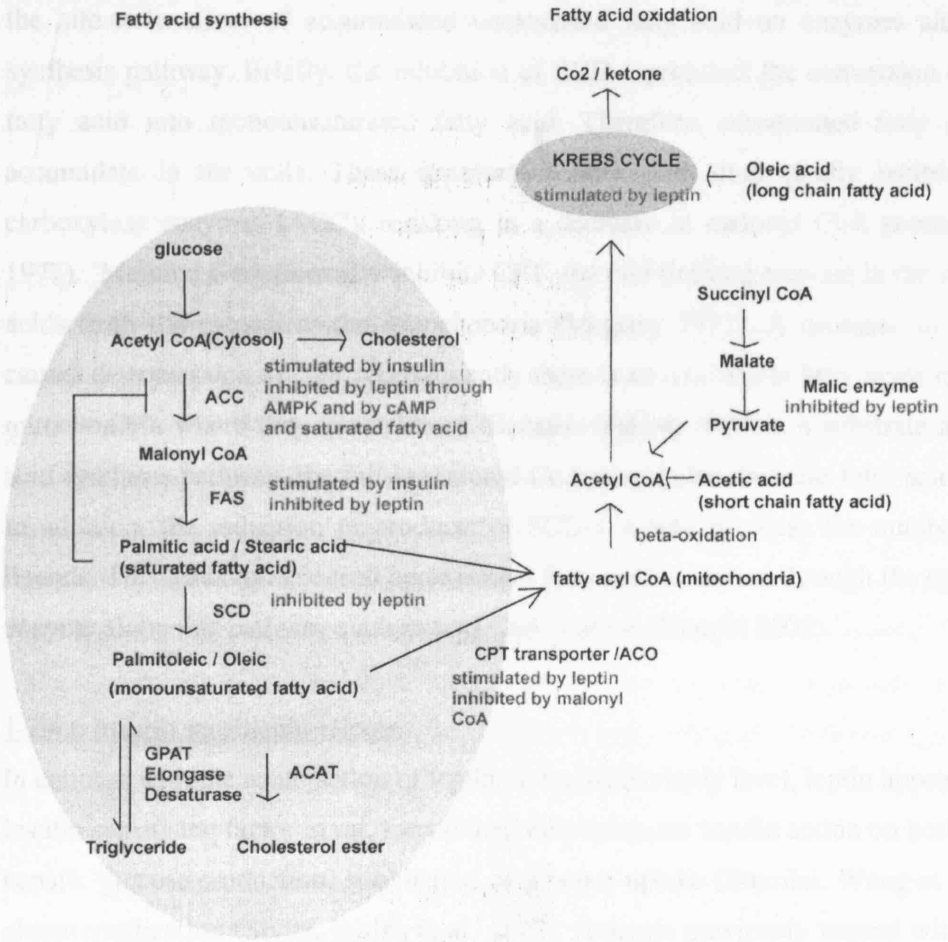
1.2.4.5 Leptin and fatty acid oxidation in non-adipose tissue

Leptin also plays an important role in the limitation of fatty acid deposit in non-adipose tissue. Adenovirus-mediated expression of leptin in rats reduces triglyceride content of islets, muscle and liver, regardless of the presence of exogenous free fatty acids (Shimabukuro, Koyama et al. 1997). In islets of wild-type mice, the reduction in triglyceride levels involves an increase in free fatty acid oxidation, a decrease in esterification and a reduction in free fatty acid synthesis (Shimabukuro, Koyama et al. 1997). Studies performed in pancreatic islets showed that leptin attenuates fatty acid synthesis by inhibiting and downregulating acetyl coA carboxylase (Shimabukuro, Koyama et al. 1997Zhou, 1997) and activates free

fatty acid oxidation by upregulating mitochondrial and peroxisomal oxidation enzymes such as CPT-1 and ACO (Zhou, Shimabukuro et al. 1997). The same study showed that leptin attenuates fatty acid esterification in islets cells by downregulating Glycerol Phosphate Acyl Transferase (GPAT) enzyme (Zhou, Shimabukuro et al. 1997).

In rat soleus muscle, both acute (Muoio, Dohm et al. 1999) and chronic leptin treatment up to 2 weeks (Steinberg, Bonen et al. 2002) increases triglyceride hydrolysis and fatty acid oxidation. Chronic changes in hydrolysis occur without changes in HSL expression and oxidative changes occur without an increase in expression of key oxidative enzyme (Steinberg, Bonen et al. 2002). In muscles, the central mediator of the oxidative effect of leptin is AMPK. Leptin stimulates phosphorylation and activation of the alpha 2 catalytic subunit of AMPK, causing the inhibition of acetyl coenzyme A carboxylase (Minokoshi, Kim et al. 2002). Reduction in the level of malonyl CoA activates CPT and this leads to an increase in transport of fatty acid into the fatty acid oxidation cascade. Stimulation of muscle fatty acid oxidation by leptin has been observed in human muscle from lean subjects.

Figure 1.14 Leptin and fatty acid metabolism



In the liver, leptin reduces hepatic liver content by repressing the expression of steroyl-CoA Desaturase-1 (SCD-1), a rate limiting enzyme in the biosynthesis of monosaturated fats such as palmitoleic and oleic acid from saturated fatty acid such as palmitic and stearic acid. The role of SCD-1 as a mediator of leptin effects has been confirmed through the increase in mRNA level of SCD-1 in ob/ob mice, the correction of this increase by leptin administration (Cohen 2002) and the correction of ob/ob phenotypes such as hypometabolism by the removal of SCD-1 gene from these animals (Cohen 2002).

The monounsaturated products of SCD-1 are the most abundant fatty acids found in triglycerides, cholesterol esters and phospholipids (Miyazaki 2000). Mice lacking SCD-1 have markedly reduced rates of both triglyceride and cholesterol ester synthesis as well as reduced levels of VLDL production (Miyazaki 2000). In the absence of SCD-1 or when the enzyme is blocked by leptin, lipid synthesis and VLDL production are blocked and there is an increase in fatty acid oxidation as shown by an increase in ketone body in SCD-1 knock out mice (Cohen 2002).

Underlying the mechanism linking SCD-1 inhibition to an increase in fatty acid oxidation is the allosteric effect of accumulated unsaturated fatty acid on enzymes along fatty acid synthesis pathway. Briefly, the inhibition of SCD-1 prohibits the conversion of unsaturated fatty acid into monounsaturated fatty acid. Therefore, unsaturated fatty acid starts to accumulate in the cells. These unsaturated fatty acid allosterically inhibits acetyl coA carboxylase enzymes (ACC), resulting in a decrease in malonyl CoA production (Lunzer 1977). Malonyl CoA normally inhibits CPT, the rate limiting enzyme in the shuttle of fatty acids from the cytosol to the mitochondria (Mcgarry 1977). A decrease in malonyl CoA causes de-repression of CPT, consequently there is an increase in fatty acids transport to the mitochondria where they are oxidised. Because malonyl CoA is a substrate along the fatty acid synthesis pathway, the fall in malonyl CoA would also decrease fatty acid biosynthesis. In addition, the reduction in products of SCD-1 would decrease the number of PPAR-α ligands. This leads to increased peroxisomal fatty acid oxidation through the up regulation of enzyme along this pathway such as acyl CoA oxidase (Ntambi 2002).

1.2.4.6 Insulin sensitising effects

In contrast with the acute action of leptin, at the whole body level, leptin appears to act as an insulin sensitising factor in rat. Leptin markedly enhances insulin action on both inhibition of hepatic glucose production, stimulation of glucose uptake (Barzilai, Wang et al. 1997) and glucose utilization (Sivitz, Walsh et al. 1997). Animals previously treated with leptin show an increase in glucose uptake and glycogen synthesis in peripheral tissues in response to

insulinemic clamp as compared with food restricted litter mates (Barzilai, She et al. 1999). Leptin increases glucose uptake, glucose decarboxylation and glycogen synthesis in incubated soleus muscle (Ceddia, William et al. 1999). It also increases glycogen synthesis in hepatocytes (Cohen, Werrmann et al. 1998). In adipocytes, chronic exposure to leptin for 15 hours increases basal and insulin induced glucose decarboxylation (Ceddia, William et al. 1998) as well as Krebs cycle activity (Ceddia, William et al. 2000).

Intracellularly, leptin can activate similar pathways to insulin. Activation of IRS-2 in hepatocytes results in inhibition of hepatic gluconeogenesis (Anderwald, Muller et al. 2002), while activation of PI3 kinase through the short form of leptin receptor results in PKB mediated activation of PDE3B and reduction of cAMP levels (Zhao, Shinohara et al. 2000). In C2C12 muscles, leptin acting via the short form of the leptin receptor mimics the insulin effect on glucose transport and glycogen synthesis through JAK2 mediated recruitment of IRS-2 and PI3 kinase (Kellerer, Koch et al. 1997). The effect is mimicked *in vivo* through leptin infusion (Kamohara, Burcelin et al. 1997; Wang, Chinookoswong et al. 1999). Leptin can modify insulin signalling by differentially activating IRS proteins. In the hepatic Fao cell line, insulin induced association of the regulatory p85 sub unit of PI3 kinase with IRS-1 is increased whereas p85 association with IRS-2 is significantly reduced after leptin pre incubation. As far as modification of insulin effects by leptin is concerned, cell type specific response and certain discrepancies may arise from this differential modification of IRS by leptin and the consequences on their downstream signalling cascade (Szanto and Kahn 2000).

1.2.4.7 Leptin resistance

Although experiments in ob/ob mice suggests that leptin administration should lead to consecutive down regulation of food intake and reduction in adipocyte mass, the negative feedback loop has not been demonstrated at physiological leptin levels in obese humans. Small doses of leptin do reduce hyperphagia and cause weight loss through body fat reduction in leptin deficient patients (Matson and Ritter 1999), but only a modest effect has been observed in obese patients without leptin deficiency (Heymsfield, Greenberg et al. 1999). On the contrary, the concentration of leptin correlates directly to body fat content and hyperleptinaemia is common in obese subjects (Frederich 1995; Hamilton 1995; Lonnqvist 1995). This observation has led to the hypothesis that leptin resistance exists in most cases of diet-induced obesity (Considine, Sinha et al. 1996).

In C57BL/6J mice fed a high fat diet, levels of hypothalamic STAT3 activation are reduced when leptin is given peripherally but the mice also show reduced response to intracerebroventricular (ICV) administered leptin (El-Haschimi, Pierroz et al. 2000). This

observation suggests that central leptin resistance occurs at two stages; during the transport of leptin across blood brain barrier and in leptin signalling cascade upstream of STAT3. The leptin labeling experiments confirms a reduction in leptin transport across blood brain barrier in obese CD-1 rodent(Banks, DiPalma et al. 1999). Plasma triglyceride levels are one of the main inhibitors of leptin transport across blood-brain barrier. Increases in plasma triglyceride levels from chronic starvation, diet induced obesity, or feeding with full fat milk, in which fats are 98% triglyceride, inhibits leptin transport across blood brain barrier (Banks, Coon et al. 2004). Initially this inhibition may arise as an anti-anorectic mechanism, but excess plasma triglyceride level resulting from excess feeding and obesity may lead to pathological leptin resistance.

Further contributions to central leptin resistance come from defects in downstream leptin signalling cascade. Dysregulation of leptin receptor signalling then manifests itself in the reduction in STAT3 activation (El-Haschimi, Pierroz et al. 2000). Studies suggest that SOCS3 is the main mediator of leptin resistance following hyperleptinaemia since it attenuates leptin signalling and its expression increases with increasing leptin concentration (Bjorbaek, El-Haschimi et al. 1999; Emilsson, Arch et al. 1999). An increase in SOCS3 mRNA level has been detected in dorsomedial hypothalamic nuclei of Agouti mice (Bjorbaek, Elmquist et al. 1998) and diet induced obese mice (Munzberg, Flier et al. 2004), which exhibited leptin resistance hyperleptinaemia and resistance to both central and peripheral leptin administration. Similar up regulation of SOCS3 expression have been observed in cultured cells (Banks, Davis et al. 2000) and in the hypothalamus of ob/ob mice administered with leptin but not in db/db mice (Bjorbaek, Elmquist et al. 1998).

Less information exists regarding leptin resistance in peripheral tissues, but a reduction in leptin stimulated fatty acid oxidation in soleus muscle of rats fed with a high fat diet over 4 weeks suggests that leptin resistance does exist in skeletal muscle (Steinberg and Dyck 2000). The presence of peripheral leptin resistance is further substantiated by the failure of leptin to stimulate fatty acid oxidation in muscles from obese humans (Steinberg, Parolin et al. 2002) and the lack of potentiation of insulin effects on glucose uptake in muscle of obese animals (Mizuno, Murakami et al. 2001). Evidence suggests that hyperinsulinaemia contributes to leptin resistance in the periphery. Insulin attenuates leptin dependent STAT3 phosphorylation in hepatoma cell line, although severe attenuation only occur when cells were pre incubated with insulin for up to 1 hour (Kuwahara, Uotani et al. 2003). In HEK293 cells, leptin stimulated JAK2 autophosphorylation is inhibited after preincubation in the presence of high insulin. The inhibitory effect may have occurred through SHP mediated dephosphorylation of JAK2 (Kellerer, Lammers et al. 2001). Otherwise, the induction of

SOCS3 expression by insulin such as those observed in adipocytes may inhibit leptin signalling (Emanuelli, Peraldi et al. 2000).

Induction of hyperleptinaemia by high sucrose diet-induced insulin resistance in mice suggests that insulin resistance may play a role in dysregulation of leptin secretion (Hintz, Aberle et al. 2003). This is supported by an increase in plasma leptin concentration in glucosamine induced insulin resistance (Wang, Liu et al. 1998). Alternatively, evidence suggests that TNFα plays a vital role in the initiation of hyperleptinaemia. TNFα treatment of 3T3-L1 adipocytes results in rapid stimulation of leptin release in a cycloheximide independent but secretion dependent manner and TNFα treatment increases plasma leptin levels in C57BL/6 mice (Kirchgessner, Uysal et al. 1997). This finding suggests that elevated adipose tissue production of TNFα in obesity (Hotamisligil, Shargill et al. 1993) may contribute to obesity related hyperleptinaemia. Indeed, correlation between body fat and leptin concentration is disrupted in obese TNFR knock out mice (Schreyer, Chua et al. 1998), implying that TNFα links the degree of adiposity to leptin production.

The paradoxes of leptin resistance and hyperleptinaemia in obesity suggest that it could be misleading to classify leptin as an anti-obesity hormone. Through out evolution obesity has not posed a selection problem. On the contrary, organisms evolve to take advantage of plentiful food supply to aid survival during time of starvation. Instead, it has been suggested that hyperleptinaemia in obesity actually helps to limit steatosis in non adipose tissues during caloric excess. In wild type mice fed high caloric diet, a 150 fold increase in body fat only lead to 2 fold increases in triglyceride content in non adipose tissues. In contrast, ob/ob, db/db and fa/fa mice have non adipose tissue triglyceride 100 times higher than controls. Controlled amounts of triglyceride in wildtype mice is achieved through activation of fatty acid oxidation and attenuation of lipogenesis in non adipose tissue such as the islets (Lee, Wang et al. 2001). Hyperleptinaemia also protects acyl CoA synthase transgenic mice from lipotoxic cardiomyopathy (Lee, Naseem et al. 2004). The protection from lipotoxicity achieved from hyperleptinaemia may account for the fact that lipotoxic complications of diet induced obesity do not occur until late in life when there is high degree of leptin resistance (Wang, Pan et al. 2001). These experiments suggest that leptin could be more accurately classified as a hormone that allows organisms to store fat in adipose tissues without the harmful effect of triglyceride accumulation in non adipose tissue during time of caloric excess.

The existence of leptin resistance also suggests that biological effects of leptin are more pronounced when leptin levels are decreasing than when circulating level of leptin is

elevated. Leptin is more effective as a starvation signal, regulating human appetite when the level decreases. In support of this view, it has been demonstrated that the sensation of hunger during prolonged consumption of a moderately energy restricted diet is correlated with reduction of plasma leptin level (Keim, Stern et al. 1998). The central role of leptin in energy homeostasis could be for reduced leptin production to function as a signal of negative energy balance and low energy reserve instead of an indicator for over supply of energy. In fact, the normal compensatory decreases of energy expenditure and thyroid axis function in response to consuming energy restricted diet in humans were prevented by low dose leptin replacement (Rosenbaum, Murphy et al. 2002), suggesting that decreases of leptin level during weight loss contribute to hunger, lower metabolic rate and weight regain.

1.2.4.8 Leptin and hypertension

One of the mechanisms linking hyperleptinaemia to cardiovascular disease is hypertension associated with hyperleptinaemia. Chronic systemic administration of leptin is found to increase blood pressure in rats (Shek, Brands et al. 1998). The effect is additive with insulin (Kuo, Jones et al. 2003). Pressor effects of leptin are blocked by adrenergic blockade, suggesting the involvement of sympathetic activity (Carlyle, Jones et al. 2002). The ability of leptin to raise blood pressure while its metabolic effects are blunted can be explained by the concept of selective leptin resistance (Mark, Correia et al. 2002). Briefly, leptin affects metabolism and the cardiovascular system through two distinct sympathetic routes. The effect on renal sympathoexcitation requires α-melanocyte-stimulating hormone while the pressor effect on brown adipose tissue, which increases thermogenic metabolism, requires corticotrophin releasing hormones (Haynes, Morgan et al. 1999; Correia, Morgan et al. 2001). In Agouti mice, the latter effect is blunted and obesity develops because of reduced thermogenesis. Concomitantly, renal mediated hypertension develops through over activation of sympathoexcitation (Rahmouni, Haynes et al. 2002). It remains unproven whether selective resistance to leptin occurs in humans; however evidence suggests that this could be the case. In human disease states that are characterised by increased sympathetic activity such as congestive heart failure and hypertension, renal norepinephrine spillover has been correlated with plasma leptin (Eikelis, Schlaich et al. 2003). Increased plasma leptin has also been correlated with increased sympathetic activation (Paolisso, Manzella et al. 2000).

1.2.4.9 Mechanism linking hyperleptinaemia to accelerated lesion formation

At the molecular level, leptin may contribute to accelerated atherosclerosis. Leptin receptors are found on the endothelium (Sierra-Honigmann 1998), macrophages and foam cell (Park 2001) and on vascular smooth muscle cells (Oda 2001). Many of its effects on these cells are

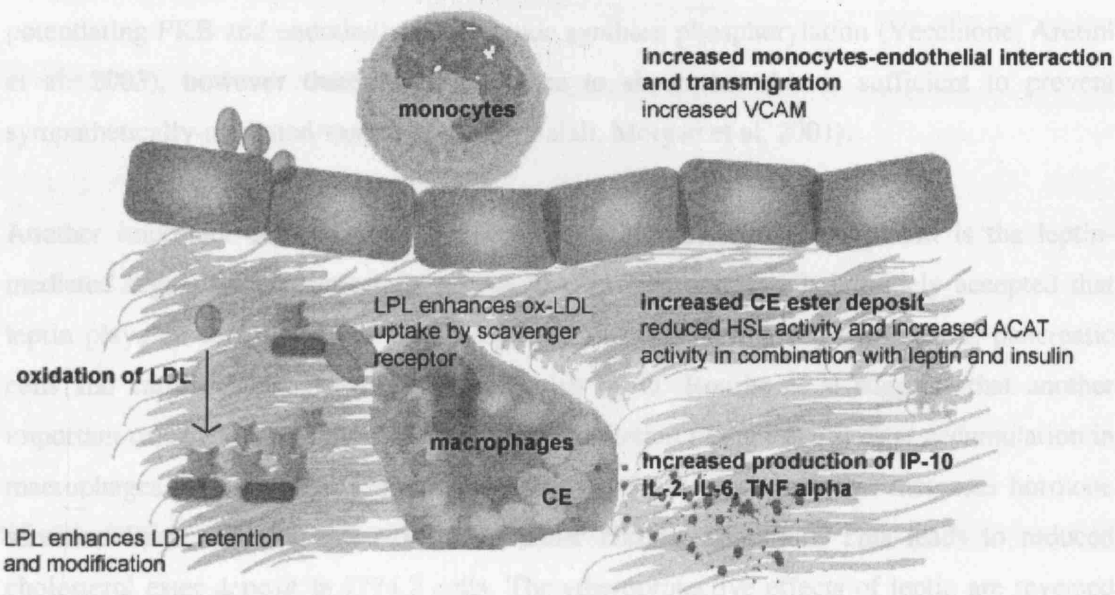
proatherogenic. For example, leptin stimulates the secretion of endothelin-1, a potent vasoconstrictor and mitogen, from endothelial cells (Quehenberger 2002). It induces migration and proliferation of endothelial cells through the increase in expression of vascular endothelial cell growth factor receptor (VEGFR-1), hence increasing the sensitivity of HUVEC to angiogenic activity of VEGF (Park 2001; Goetze 2002). Leptin induces migration of vascular smooth muscle cells in a PI 3-Kinase dependent manner (Oda 2001). It also contributes to matrix remodelling by inducing the expression of matrix metalloproteinases in endothelial cells (Park 2001). High concentrations of leptin found in obesity, cause coronary endothelial dysfunction, attenuating coronary dilator response to acetyl choline (Knudson 2005). Levels of leptin also correlate well with vascular calcification, a hallmark of atherosclerosis. In fact, leptin actively promotes osteoblastic differentiation and calcification of vascular cells *in vitro* (Parhami 2001).

Many effects of leptin result from an increase in oxidative stress. For example, leptin increases mitochondrial superoxide production in bovine aortic endothelial cells by increasing fatty acid oxidation via PKA activation in an additive manner with glucose, leading to an up regulation of MCP-1, a potent inducer of monocytic cells recruitment (Yamagishi 2001). Leptin increases the accumulation of ROS in human umbilical vein endothelial cells (HUVEC), increases JNK activity, AP-1 binding, activation of NFk-B (Bouloumie 1999). In a different study leptin administration decreases the activity of antioxidant enzymes contained in plasma lipoprotein, the paraoxonase 1 (PON1), and platelet activating factor-acetylhydrolase (PAF-AH), resulting in an increase in oxidative stress (Beltowski 2003). Injection of leptin over a 7 day period in mice increases lipid peroxidation products, reduces urinary excretion of nitric oxide metabolite and increases renal sodium retention. NO deficiency and sodium retention may contribute to leptin-induced hypertension (Beltowski, Wojcicka et al. 2004).

Further contributions of leptin to proatherogenic activity depend on the proinflammatory role of leptin. Inflammation is found to be associated with cardiovascular disease in several studies employing high sensitive C-reactive protein (hs-CRP) as a marker (Ridker 1999; Ridker 2001; Rifai 2001). Although none of the studies focused on patients with diabetes, the finding that inflammatory markers also predict the incidence of diabetes (Festa 2002) has led to the hypothesis that inflammation could be the common link between both diseases. The leptin level is found to correlate with C-reactive protein in the degree of inflammation in patients with diabetes (Mohamed-Ali 1998; Van Dielen 2001; Bullo 2003). Because leptin increases as part of the acute phase response (Wallace 2000), hyperleptinaemia could accompany low grade inflammation in chronic inflammatory disease such as atherosclerosis.

CRP itself is found to promote monocyte chemotaxis, cytokine release and tissue factor secretion. In endothelial cells, CRP inhibits eNOS and stimulates adhesion molecules (VCAM and ICAM) and adhesion of monocyte to endothelial cells (Venugopal 2002).

Figure 1.15 Proatherogenic effects of leptin in macrophages



Genetic studies of rodent with ob/ob or db/db mutation links leptin directly to an up regulation of inflammatory immune responses (Loffreda 1998). In ob/ob mice, a chronic inflammatory disease such as arthritis is attenuated (Busso 2002) and inhibition of leptin secretion reduces colonic inflammation in rat (Barbier 2001).

Many of the proinflammatory mechanisms of leptin resulted from direct effect of leptin on monocytes/macrophages. Leptin potentiates immune cells recruitment by up regulating vascular adhesion molecule-1 (VCAM-1)(Porreca 2004). In an experiment in vitro, macrophages from control mice exhibit an up regulation of TNF, IL-12, IL-6 as well as phagocytosis in response to LPS when administered high doses of leptin (Loffreda 1998). When incubated with leptin, increases in expression of TNF-α and IL-6 have been observed in resting $PBMC_{[t1]}$ and monocytes. Prolonged incubation also increases IFN--γ in PBMC (Zarkesh-Esfahani 2001). Leptin strongly enhances the expression and secretion of the interferon -γ inducible protein (IP-10), a proinflammatory cytokine, in human monocytic cell line as well as in human peripheral mononucleolar cells (Meier 2003). Finally in J774A.1, leptin increases macrophage activation in response to interferon -γ activation (Raso 2002).
On the other hand leptin is found to induce expression and secretion of IL-1Ra, an anti-inflammatory cytokine, by human monocytes *in vitro* (Gabay 2001) and macrophages from

ob/ob mice exhibit increased sensitivity to proinflammatory cytokines (Faggioni 1999). Lower levels of anti-inflammatory cytokines such as IL-10, IL-6 and IL-1ra and higher levels of proinflammatory cytokines such as IL-12, IL-18 and interferon-γ are detected after LPS in ob/ob mice (Faggioni 1999; Guebre-Xabier 2000). In conjunction with insulin, leptin has been found to promote vasodilation by promoting endothelial nitric oxide release by potentiating PKB and endothelial nitric oxide synthase phosphorylation (Vecchione, Aretini et al. 2003), however there is no evidence to show that this is sufficient to prevent sympathetically-mediated vasoconstriction (Jalali, Morgan et al. 2001).

Another important contribution from leptin to atherosclerosis development is the leptin-mediated regulation of cholesterol metabolism in macrophages. It is widely accepted that leptin plays an important role in the regulation of triglyceride deposit in liver, pancreatic cells and cardiac cells. A study from this lab by O' Rourke et al suggests that another important role of leptin in the periphery is the limitation of cholesterol ester accumulation in macrophages under normal glucose condition. At 5mM glucose, leptin increases hormone sensitive lipase activity and expression under chronic condition. This leads to reduced cholesterol ester deposit in J774.2 cells. The atheroprotective effects of leptin are reversed under high glucose levels. Here, leptin becomes proatherogenic. At 20mM glucose concentration, leptin decreases HSL activity and expression whilst it increases the activity of ACAT enzyme. Together leptin increases cholesterol ester deposition in J774.2 cells under high glucose condition (O'Rourke 2002).

Finally, leptin increases the expression of lipoprotein lipase, a proatherogenic enzyme, in J774 macrophages via a PKC and oxidative stress dependent mechanism (Maingrette 2003). Proatherogenic effects of macrophage lipoprotein lipase have been confirmed *in vivo*: atherosclerosis is reduced in mice with macrophage-specific lipoprotein lipase deletion (Babaev 1999) and atherosclerosis is enhanced in apoE-/- mice with macrophage-specific lipoprotein overexpression (Wilson 2001). Lipoprotein lipase enhances macrophage cholesterol ester uptake through two main mechanisms. First it acts as a bridge between LDL and proteoglycan, lengthening resident time of LDL in the intima, thus allowing to be modified much more extensively. Secondly, it links LDL or modified LDL particles to cell surface receptors, facilitating the uptake into macrophages (Pentikainen, Oksjoki et al. 2002).

1.2.5 Hyperglycaemia

1.2.5.1 Association between hyperglycaemia and cardiovascular disease

Since 1993 several prospective studies summarised in table 1.1 have shown that hyperglycaemia is a cardiovascular risk factor across all ethnic groups. Studies which indicate that cardiovascular complications are already frequent at the diagnosis of type 2 diabetes only confirm this association since impaired glucose tolerance (IGT), a condition with near normal fasting plasma blood glucose and elevated postprandial glucose level, usually occurs several years before the onset of full blown diabetes ((WHO) 1980). Lowering of glucose levels in diabetic patients has been associated with lowered risk from cardiovascular disease. In the UKPDS trial, intensive treatment of hyperglycaemia using sulfonylurea or insulin resulted in a significant reduction in microvascular and macrovascular complications (UKPDS 1998). Regression of carotid intima media thickness has been observed with reduction of postprandial hyperglycaemia in Type 2 diabetic patients (Esposito 2004) and clinical outcome after acute coronary syndrome improved with glycaemic control strategies in Type 2 diabetes (McGuire 2004).

1.2.5.2 Pathology of hyperglycaemia induced damage

A range of mechanisms have been suggested by which hyperglycemia might contribute to the development of atherosclerosis. To start with, hyperglycaemia accelerates endothelial dysfunction. Mechanistically, high levels of glucose inhibit production of nitric oxide in arterial endothelial cells (William 1998) and stimulate production of plasminogen activator inhibitor-1 (PAI-1)(Du 2000). Hyperglycaemia also reduces heparin sulphate glycosaminoglycans through modification of molecular structure, leading to changes in arterial wall extracellular matrix component and increased atherosclerosis susceptibility (Vogl-Willis 2004) through increased tranvascular LDL transport (Kornerup 2003). High glucose also promotes oxidation pathway dependent oxidative modification of these LDL, turning them into more potent activators of atherogenesis (Kawamura, Heinecke et al. 1994).

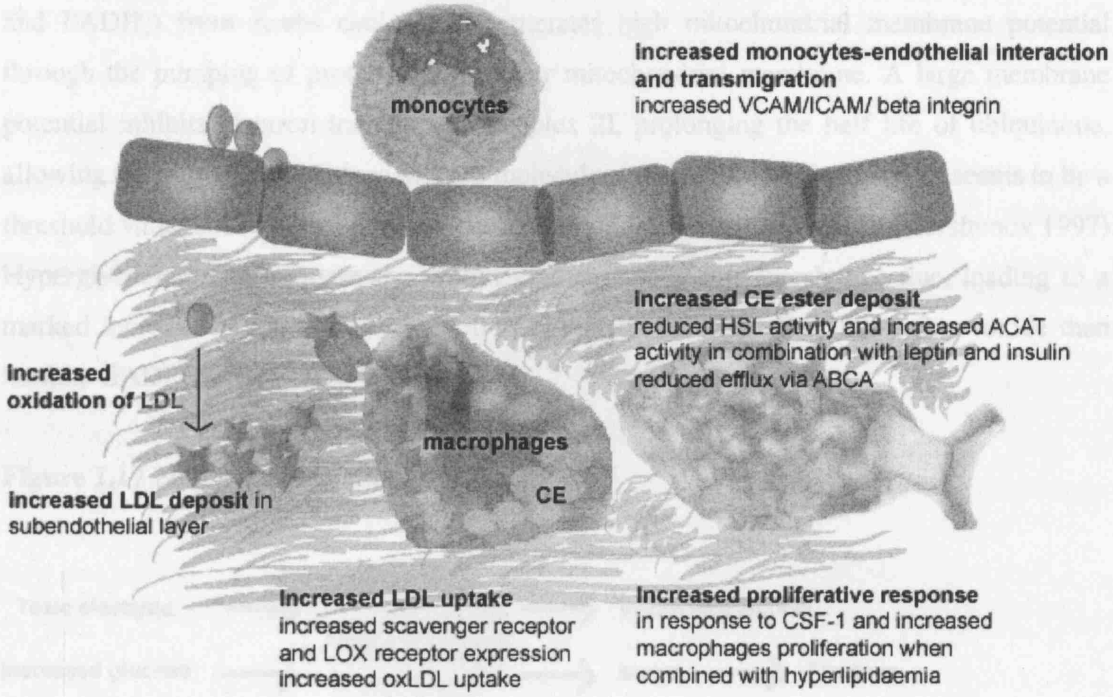
At the later stages in foam cell formation, hyperglycaemia enhances leukocyte-endothelial (Morigi 1998) and neurophils-endothelial interaction by increasing the expression of ICAM, P-selectin and E-selectin (Omi 2002) as well as beta 2 integrin subunit on monocytes (Fogelstrand 2004) and VCAM-1 and ICAM-1 on endothelial cells(Altannavch 2004). High glucose also increases monocyte binding to human aortic endothelial cells (Ikeda U 1998) and monocyte transmigration through endothelial cells (Rattan 1996). An increase in MCP-1 levels has been observed in diabetic patients (Piemonti 2003). In macrophages, hyperglycaemia leads to an increase in proliferative response to colony stimulating factor-

1(Liu 1995) by enhancing CSF-1 receptor mRNA level up to threefold (Saini 1996). Interestingly, the combined effect of hyperglycaemia and hyperlipidaemia increases macrophage proliferation in lesions of LDL-R deficient mice fed with cholesterol rich diet (Lamharzi 2004), but there is no effect from glucose alone. Finally, hyperglycemia also affects intracellular cholesterol homeostasis. It potentiates cholesterol ester accumulation in macrophages through upregulation of scavenger receptor (Fukuhara-Takaki K 2004) and increased expression of Lectin-like oxidized LDL receptor-1 (LOX-1), a newly discovered receptor for oxidized LDL(Li 2004). It increases ACAT activity and decreases HSL activity when combined with hyperinsulinaemia and hyperleptinaemia(O'Rourke 2002). Accumulation of lipid in macrophages then promotes elevated secretion of proinflammatory cytokines including TGF-beta, TNF-α, IL-1, IL-6, IL-8 and M-CSF (van Reyk and Jessup 1999).

Table 1.1 Hyperglycaemia and incidence of cardiovascular disease

counry	year	size	age	time	End point	Reference
Finland	1993	133	45-64	10	CHD mortality	(Uusitupa 1993)
Finland	1994	229	65-74	3.5	CHD mortality morbidity	(Kuusisto 1994)
Finland	1994	229	65-74	3.5	Stroke incidence	(Kuusisto 1994)
Sweden	1995	411	23-94	7	CHD mortality morbidity	(Andersson 1995)
US	1995	1370	>30	10	CHD mortality and stroke	(Klein 1995)
Denmark	1995	328	20-65	5	CHD mortality	(Gall 1995)
Germany	1996	1139	30-55	11	CHD mortality	(Hanefeld 1996)
Germany	1996	290	<76	10	CHD mortality	(Standl 1996)
Finland	1996	1059	45-64	7	Myocardial infarction	(Lehto 1996)
Finland	1997	1059	45-64	7	CHD mortality morbidity	(Lehto 1997)
UK	1998	2693	25-65	8	CHD mortality morbidity	(Turner 1998)
US	1998	471	25-64	7.5	CHD mortality	(Wei 1998)

Figure 1.16 Proatherogenic effects of hyperglycaemia on macrophages



1.2.5.3 Mechanisms of hyperglycaemia-induced damage

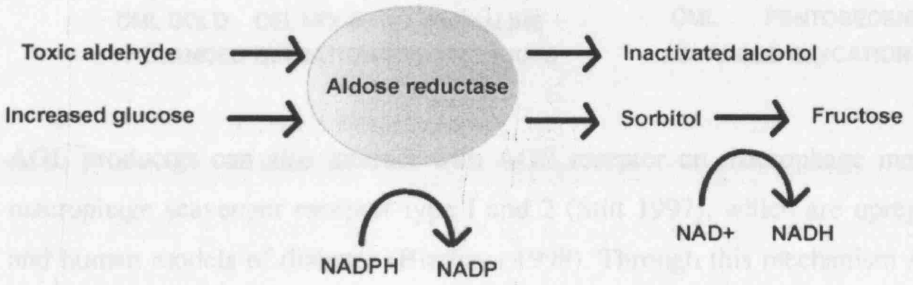
Evidence suggests that the proatherogenic role of hyperglycaemia is a consequence of an increase in polyol pathway flux and hyperglycaemia-induced overproduction of superoxide by mitochondrial electron transport chain (Nishikawa 2000). Both phenomena inhibit glyceraldehyde-3-phosphate dehydrogenase (GADPH) enzymes (Du 2000), causing a diversion of upstream glycolytic intermediates into three major pathways mediating hyperglycaemia-induced damage including the formation of AGE, hexosamine pathway flux and overactivation of PKC isoforms (Du 2003).

1.2.5.3.1 Polyol pathway flux: In the polyol pathway shown in figure 1.17, Aldose Reductase catalyses the NADPH-dependent reduction of carbonyl compounds including glucose. Aldose reductase has low affinity for glucose (high Km) and at normal glucose concentration this pathway only accounts for a very small percentage of glucose utilisation in the cell. Hyperglycaemia increases sorbitol production, with a consequent reduction in NADPH concentration and an increase in NADH concentration. In the polyol pathway sorbitol is oxidised to fructose by the enzyme sorbitol dehydrogenase, with NAD+ reduced to NADH. An increase in the intracellular NAD:NADH ratio inhibits GADPH, causing an increase in concentration of triose phosphate (Williamson 1993).

1.2.5.3.2 Overproduction of superoxide: Normally superoxide is generated in the mitochondrial electron transport chain by free radical intermediates of coenzyme Q, also

known as ubiquinone. Hyperglycaemia causes an overproduction of electron donors (NADH and $FADH_2$) from Krebs cycle. This generates high mitochondrial membrane potential through the pumping of protons across inner mitochondrial membrane. A large membrane potential inhibits electron transport at complex 2I, prolonging the half life of ubiquinone, allowing the conversion of more oxygen molecules into superoxide ions. There seems to be a threshold value above which superoxide production is markedly increased (Korshunov 1997). Hyperglycaemia can increase the proton gradient above this threshold value, leading to a marked increase in superoxide production in endothelial cells (Du 2001). Superoxide then inhibits GADPH activity (Du 2000).

Figure 1.17 Polyol pathway flux

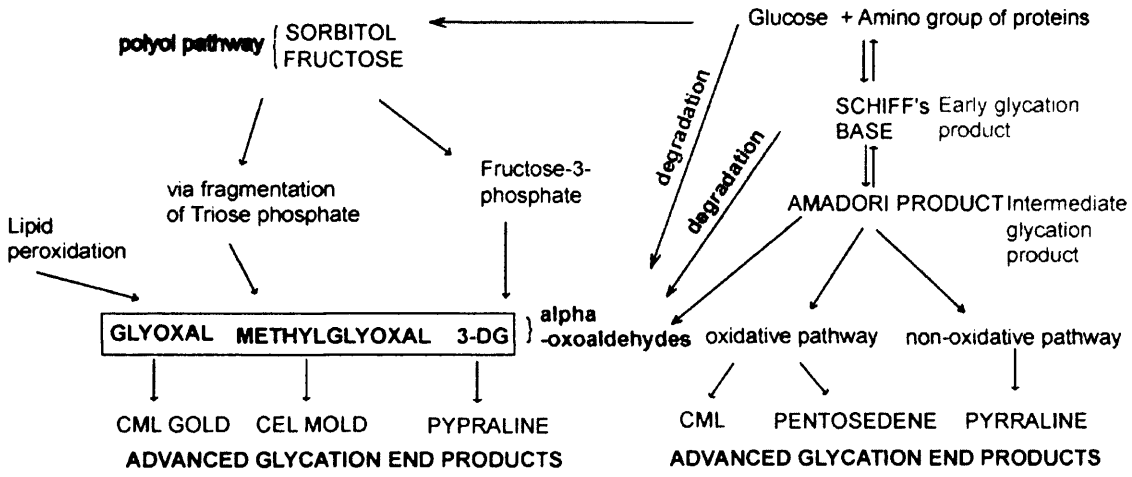


1.2.5.3.3 Formation of advanced glycation end product (AGE). Hyperglycaemia can enhance non-enzymatic glycosylation of lipoproteins, apolipoproteins and clotting factors. Intracellular AGE forms at a rate up to 14-fold faster in high glucose condition (Giardino 1994). Increasing amounts of triose phosphate as a result of GADPH inhibition provides more precursor for methyglyoxal-derived AGE, the primary intracellular AGE induced by hyperglycaemia (Shinohara 1998). Through a complex series of dehydration and oxidation reactions shown in Figure 1.18, the formation of advance glycation end products occurs. AGE can also be derived from auto-oxidation of glucose to glyoxal (Wells-Knecht 1995), decomposition of Amadori product (glucose-derived 1-amino-1-deoxyfructose lysine adducts) to 3-deoxyglucosome and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methyglyoxal (Thornalley 1990). The rate of AGE formation from glucose-derived dicarbonyl precursors generated intracellular is faster than glucose itself, hence it is likely that intracellular hyperglycaemia is the primary event in the formation of AGE (Raj 2000).

AGE modifications have a number of pathological consequences that impact on the development of atherosclerosis. One important one is the effect on LDL. AGE-apo B levels are 4-fold higher in diabetic patients (Bucala 1994) (Stitt 1997). Clearance of these modified

LDL particles via LDL receptors is much slower (Bucala 1994). This may lead to hyperlipoproteinaemia and facilitation of AGE-oxidised LDL deposition in the vessel wall.

Figure 1.18 AGE formation



AGE productgs can also interact with AGE receptor on macrophage membranes such as macrophage scavenger receptor type I and 2 (Stitt 1997), which are upregulated in animal and human models of diabetes (Bierhaus 1998). Through this mechanism AGE can activate intracellular signalling cascades involving p21ras/MAPK and activation of NF-kB (Schmidt 1994; Yan 1994; Lander 1997). The cascade upregulates pro-inflammatory cytokines and growth factors such as TNF-α, IL-6, macrophage colony stimulating factor-1, granulocyte-macrophage stimulating factor, insulin like growth factor-1 (Kirstein 1992) and platelet-derived growth factor (Doi 1992) by macrophages and mesangial cells. AGE increases expression of pro-coagulatory and pro-inflammatory molecules such as thrombomodulin and VCAM1 by endothelial cell (Vlassara 1988; Morohoshi 1995; Abordo 1996; Bierhaus 1998). The link between AGE and atherosclerosis is confirmed through the reduction of macrovascular disease in atherosclerosis prone type 1 diabetic mouse model in a glucose and lipid-independent fashion after a blockade of AGE receptor (Park 1998).

1.2.5.3.4 Over-activation of PKC. PKC's are a family of 12 diacylglycerol (DAG)-sensitive serine/threonine kinase, divided into three families – conventional, novel and atypical- according to their calcium and phospholipid dependence. When activated PKC isozymes can translocate from cytosol to membrane (Nishizuka 1995). Several studies have demonstrated that intracellular hyperglycaemia increases the amount of DAG in cultured smooth muscle cells(Inoguchi T 1992), aorta and heart(Inoguchi T 1992), retina (Craven 1990) and renal glomeruli (Craven 1990)of diabetic rat. This is achieved by increasing *de novo* synthesis from the glycolytic intermediate dihydroxyacetone phosphate, through

reduction of the latter to glycerol-3-phosphate and stepwise acylation (Koya 1998). Alteration in DAG level as a result of hyperglycaemia can only be corrected with a long period of intensive glycaemic control (King 1996).

PKC, beta-2 seems to be selectively increased by hyperglycaemia (Inoguchi T 1992). It is activated in large vessels in the presence of diabetes (Koya 1998) and in vascular smooth muscle cells model (Inoguchi T 1994; King 1996). However, increases in alpha and epsilon isoform of PKC have also been found (Koya 1998). Overall, PKC activity is increased in the retina(Shiba 1993), aorta and heart(Inoguchi T 1992), renal glomeruli of diabetic rat (Craven 1990), cultured vascular cells or tissue exposed to elevated level of glucose(Craven 1990; Williams B 1992) as well as circulating monocytes(Ceolotto 1999). The latter cells express conventional PKC isoforms, alpha and beta(Chang 1993).

Increases in PKC activity have been associated with a number of features of coronary artery disease including decreased production of nitric oxide by smooth muscle cells (Ganz 2000) and the accelerated proliferation (Patel 1999) that is caused by hyperglycaemia. It also inhibits insulin-stimulated expression of mRNA for endothelial nitric oxide synthase (eNOS) in cultured endothelial cells (Kuboki 2000) while increasing the expression of endothelin-1, a potent vasoconstrictor (Koya 1998). PKC mediates the increase in endothelial and smooth muscle cell permeability to LDL observed in high glucose (Hempel 1997; William B. 1997). Hyperglycaemia-induced activation of PKC has also been implicated in the overexpression of PAI-1 in vascular smooth muscle cells (Feener 1996; Suzuki 2002) and the activation of NF-kB in cultured endothelial cells (Pieper 1997)as well as in vascular smooth muscle cells (Yerneni 1999). In human macrophages, PKC, mitogen-activated protein kinase and NF-kB mediated the hyperglycaemic induction of Lectin-like oxidized LDL receptor-1 (LOX-1). This newly identified receptor for oxidized LDL has been classified as an essential part for human foam cell formation (Li 2004). Raised PKC activity is associated with enhanced initial monocyte adhesion to the vascular wall and fibrinogen binding (Kreuzer J 1996), their transmigration (Rattan 1996), as well as their differentiation into macrophages (Kalra 1996). In particular, the binding of monocytes to human aortic endothelial cells is enhanced in diabetes (Ikeda U 1998). A specific PKC inhibitor, Staurosporine markedly decreases the number of leukocytes adhering to high glucose treated cells (Morigi 1998). In both THP-1 monocytes and human monocytes from diabetic patients, PKC is involved in glucose induction of superoxide anion production (Venugopal 2002). This effect is complimented by an induction of reactive oxygen species production by NAD(P)H oxidase via a PKC-dependent pathway in cultured vascular cells(Inoguchi 2000).

1.2.5.3.5 Hexosamine Biosynthesis pathway

Around 2-3% of glucose-derived fructose-6-phopshate is transformed into glucosamine-6-phosphate, a substrate for the formation of glycoprotein and glycolipids (Marshall, Bacote et al. 1991). Hexosamine biosynthesis is essentially an irreversible reaction. The first step of the reaction is catalysed by Glutamine fructose-6-phosphate amidotransferase (GFAT), a rate limiting enzyme in the pathway. GFAT catalyses a reaction where glutamine donates an amino group to D-fructose-6-phosphate to produce one molecule of glutamate and one molecule of glucosamine-6-phosphate. Subsequently, UDP-N-acetyl glucosamine (UDP-GlcNAc) and other nucleotide hexosamines are formed. N-acetyl glucosamine is best recognised as a component of complex polysaccharide structure on membrane bound or secreted proteins. These proteins are synthesised in the endoplasmic reticulum and Golgi apparatus. The luminal or extracellular localization of these glycans restricts their potential for dynamic responsiveness to signals. However, N-acetyl glucosamine is also added to serine and threonine residues of nucleocytoplasmic protein through o-linked glycosylation. The simple addition of monosaccharide is catalysed by O-GlcNAc transferase (OGT).

OGT is a holoenzyme consisting of two 110kDa subunits and one 78 kDa subunit. It migrates with molecular weight of 340 kDa, indicating that the enzyme exists in a heterodimer complex. It has low Km for UDP-GlcNAc (0.545 μM) with the resulting UDP product strongly inhibiting its activity (Haltiwanger, Blomberg et al. 1992). The enzyme itself is modified by glycosylation and phosphorylation; hence it is under hormonal regulation. The expression of the 78kDa subunit is tissue specific while the 110kDa is homogeneous, suggesting that similar regulatory mechanism analogous to the p110 and p85 subunits of PI3-kinase may apply to this enzyme (Kreppel, Blomberg et al. 1997). It is vital for development as deletion of OGT locus in mice results in embryonic lethality (Shafi, Iyer et al. 2000). OGT is recruited into a complex with mSin3A and histone deacetylase through its TRP domain (Yang, Zhang et al. 2002) while deletion studies showed that the active site lies in the C terminal domain (Yang, Zhang et al. 2002).

Subsequent to the addition, O-GlcNAc residues can be removed from proteins by O-GlcNAcase enzymes such as β-N-acetylglucosaminidase or nuclear cytoplasmic O-GlcNAcase and acetyltransferase (NCOAT). The former enzyme has nucleocytoplasmic distribution. It is a monomeric enzyme with molecular weight of 130kDa but it migrates at 600kDa. This indicates that in the cell, the enzyme complexes with other proteins such as heat shock protein hsp110, hsp70, cullin, TATA binding protein 120, calcineurin, amphiphysin and dihydropyrimidinase related proteins (Wells, Gao et al. 2002). The latter enzyme, NCOAT, also possess acetyl transferase activity *in vitro* for a synthetic histone

substrate tail as well as for free core histones and reconstituted oligonucleosome substrates. HAT activity lies in the C-terminal domain while O-GlcNAcase activity lies in the N terminal domain. It contains the active site core architecture with same arrangement of alpha helices and beta sheets as other acetyltransferases. The same motif pattern is conserved in drosophila, rat, human, anopheles and *C.Elegans* (Toleman, Paterson et al. 2004).

O-linked glycosylation usually competes for the same residue as phosphorylation, especially sites specific for GSK3 (Kamemura, Hayes et al. 2002). Phosphorylation and glycosylation are reciprocal on many proteins such as Sp-1 (Haltiwanger, Grove et al. 1998), C-terminal domain of large sub-unit of RNA polymerase 2, c-Myc (Chou, Hart et al. 1995) and murine estrogen receptor-β (Cheng, Cole et al. 2000). The effect is global as okadaic acid treatment increases total amount of O-linked modification in cell lines (Lefebvre, Alonso et al. 1999). The OGT enzyme has been found on the same complex with phosphatases, therefore some sites could be transformed directly from phosphorylation to glycosylation without remaining unmodified. Glycosylation also inhibits phosphorylation at adjacent sites, possibly through steric hindrance. This inverse relationship between glycosylation and phosphorylation suggests that o-linked glycosylation could be an intracellular event that links the magnitude of signalling response to extracellular stimulus to cellular nutritional status. Glycosylation seems to limit the magnitude and timespan of phosphorylation, as shown by hyperphosphorylation of Tau and overactivation of PKB when OGT is deleted.

Modification by O-linked glycosylation contains all the hallmarks of posttranslational modifications involved in signal transduction. The modification is dynamic, its half-life much shorter than that of the modified peptide chain (Favreau, Worman et al. 1996). The addition and removal is responsive to extracellular stimuli (Kearse and Hart 1991; Kamemura, Hayes et al. 2002). OGT enzyme interacts with many scaffolding proteins, indicating that substrate specificity, localisation and activity of this enzyme are likely to be regulated by signal transduction cascades. The importance of O-linked glycosylation is confirmed by lethality of OGT knockout in mouse embryonic stem cells (Shafi, Iyer et al. 2000) and the high degree of conservation of OGT from *C Elegans* to humans (Gao, Wells et al. 2001).

1.2.5.3.5.1 Control of gene expression

O-linked glycosylation of proteins links hexosamine pathway flux with the control of transcription. Proteins modified by O-GlcNAc are highly concentrated on the nuclear pore (Hanover, Cohen et al. 1987) and on protein associated with chromatin. Predictably, transcription factors form a large proportion of glycosylated proteins. Addition of O-linked

N-acetyl glucosamine residue alters many aspects of transcription factor function. First, O-linked glycosylation **alters transcriptional activity**. One of the well characterised examples is p53. The carboxy terminus of p53 contains a basic region, which represses DNA binding by interrupting intramolecular interactions. Pab421, an antibody against the basic region, can relieve the repression. Interestingly, in EB-1 cell line, Pab421 fails to recognise the basic epitope and these p53 molecules are highly active in a DNA binding assay. Following digestion of western blot of EB-1 cell extract with hexosaminidase, the epitope became recognisable again by Pab421 antibody. Subsequently, glycosylation is detected on p53 by wheat germ agglutinin chromatography and galactosyl-transferase labelling. Together, both observations suggest that glycosylation residues lie within the basic inhibitory domain. Glycosylation of p53 in this domain disrupts an inhibitory intramolecular interaction, allowing p53 to form a stable complex with target DNA. Consequently there is an increase in p53 activity (Shaw, Freeman et al. 1996).

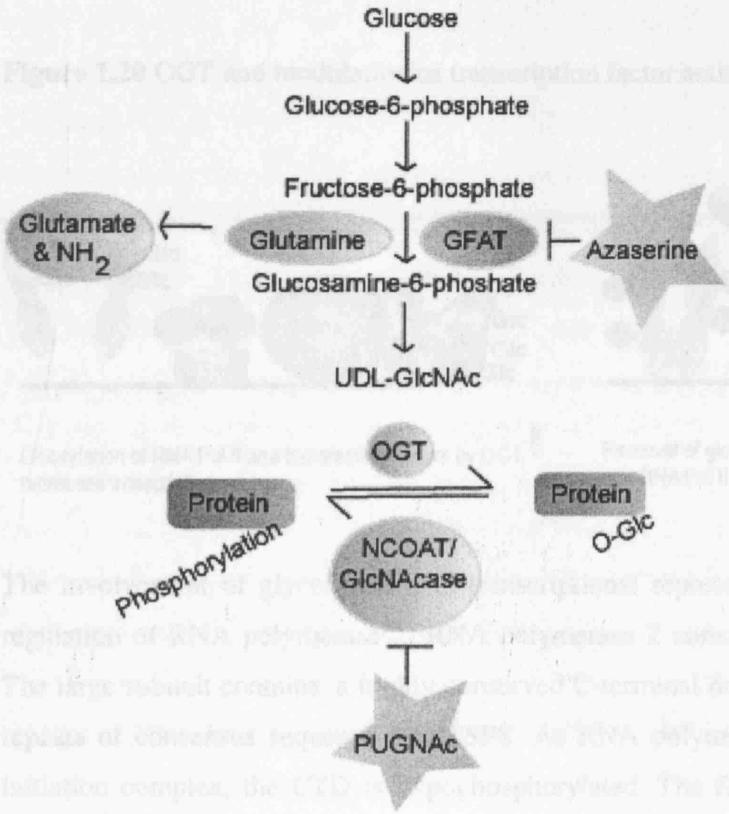
STAT5 is another protein positively regulated by glycosylation. Glycosylation of Thr92 residue is essential for STAT5 interaction with coactivator of transcription CBP, a protein with intrinsic histone deacetylase activity. Mutant STAT5 with a Thr-Ala mutation abolishes the interaction (Gewinner, Hart et al. 2004). In this case, O-link glycosylation acts as a signal for complex formation and transcriptional activation in response to nutritional status.

A number of studies suggest that Sp1 is a transcription factor that mediates effects of hexosamine flux. In bovine aortic endothelial cells, an increase in plasminogen activator inhibitor-1 expression in response to hexosamine flux depends on Sp-1 sites (Du 2000). Sp-1 in these cells are highly glycosylated in response to high glucose or glucosamine (Du 2000) and its transcriptional activation domain fused to GAL4 DNA binding domain showed increased activity in response to glucose or glucosamine (Goldberg, Whiteside et al. 2002). In the liver, glycosylation of Sp-1 is important for nuclear recruitment. Although both glucagon and insulin increase total Sp-1 level, only insulin stimulates glycosylation of Sp1, consequently only insulin induces Sp-1 nuclear recruitment and activation of calmodulin gene (Majumdar, Harmon et al. 2003). Finally, in Caco-2 cells, both glucosamine and glutamine treatment increase Sp-1 DNA binding, translocation to the nucleus and o-linked glycosylation of sp1 (Brasse-Lagnel, Fairand et al. 2003; Majumdar, Wright et al. 2004).

On the other hand, glycosylation has been implicated as a global mechanism for transcriptional repression. Chromatin immunoprecipitation experiments involving estrogen target genes such as EB1 and cathepsin D revealed that transcription factors bound to silenced areas of chromatin under estrogen depletion are hyperglycosylated (Yang, Zhang et

al. 2002). Activity of OGT in this complex is modulated by the availability of its substrate, UDP-GlcNAc and through phosphorylation. Hence OGT-mediated repression may be subjected to nutritional and extracellular hormones regulation. OGT enzyme can be recruited to specific sites on a promotor through direct interaction with mSin3(Yang, Zhang et al. 2002), a transcriptional repressor found in complex with histone deacetylases. OGT then glycosylates transcription factors at sub-stoichiometric levels. Glycosylation then leads to disruption of intramolecular forces between transcription factor complexes and inhibition of gene expression.

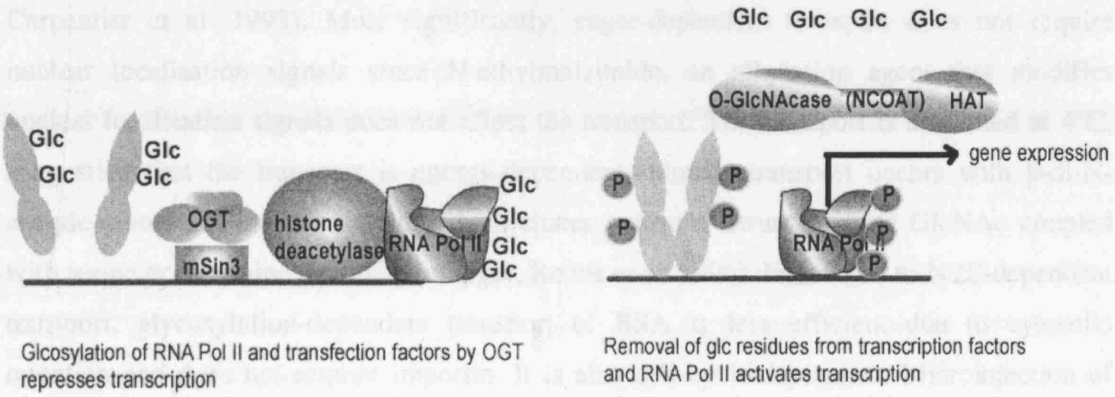
Figure 1.19 Hexosamine biosynthesis pathway



Paradoxically, disruption of Sp1 DNA binding domain has been studied most extensively. Expression of OGT directly inhibits Sp-1 mediated gene expression even though Sp-1 is not found in OGT-mSin3-HDAC complex (Yang, Zhang et al. 2002). Sp1 contains two glutamine-rich activation domains and a zinc finger DNA binding domain on the C terminus. The second glutamine-rich domain is involved with homomultimerisation of Sp1 and in the interaction with the TF_2D through TATA binding protein associated factor 2 110. The former interaction is important for synergistic activation of transcription by Sp1 and the latter is involved in the interaction of Sp1 with DNA Pol II dependent transcription. An *in vitro* pull down assay involving peptide containing the glutamine-rich domain showed that interaction with TATA binding protein associated factor 2 110 is inhibited by O-linked glycosylation of the domain (Roos, Su et al. 1997). An *in vitro* transcription system and transfection of the glutamine-rich domain into pancreatic beta cells confirmed that glycosylation of this domain indeed inhibits the ability of the domain to activate transcription (Yang, Su et al. 2001).

The discovery of OGT within the repressor complex may explain how activators and repressors that coexist on a promoter counteract each other when transferring from transcriptional activation to transcriptional repression. Traditionally, it is believed that chromatin remodelling by chromatin-remodelling factors drive nucleosome mobilisation and the removal of acetyl group from histone tails by histone deacetylases (HDAC) creates a repressive chromatin environment, limiting transcription factor access to the promotor. The view has been challenged by the recent observation that TATA binding proteins, RNAP2 and Sp1 constitutively occupy genes in silenced chromatin (Sekinger and Gross 2001). There is a possibility that these complexes remain inactive as long as their hydrophobic interactions are disrupted by o-linked glycosylation. They only become activated once the O-GlcNAc residues are removed.

Figure 1.20 OGT and modulation of transcription factor activity



The involvement of glycosylation in transcriptional repression is further supported by its regulation of RNA polymerase 2. RNA polymerase 2 consists of at least 10 polypeptides. The large subunit contains a highly conserved C-terminal domain containing as many as 52 repeats of consensus sequence YSPTSPS. As RNA polymerase 2 enters the transcription initiation complex, the CTD is hypophosphorylated. The formation of complete initiation complex leads to hyperphosphorylation on serine and threonine residue by CTD specific kinase, signalling the elongation phase of transcription. Hypophosphorylated CTD is highly glycosylated on serine and threonine residue, suggesting that glycosylation of CTD may prevent premature association of RNA polymerase 2 elongation factor or mRNA processing factor (Cervoni, Turano et al. 1997).

Another extensively studied function of O-linked glycosylation is the control of **nuclear transport**. Bidirectional exchange between the cytoplasm and nucleus in eukaryotic cells is mediated by the nuclear pore complex, a supramolecular structure of $125x10^6$ Da that is

anchored in the nuclear envelope. Nuclear pore complexes are formed from 200 proteins and involve fusion of the outer lipid bilayer and the inner lamina membrane. Proteins with molecular mass below 40kDa can cross the nuclear pore complex through passive diffusion, whereas proteins with molecular mass above 40kDa require chaperone-mediated energy consuming transport. Chaperone mediated transport occur in two steps, namely binding of nuclear localisation signal on protein to docking complex, translocation and ATP dependent dissociation of protein in the nucleus.

In 1993, Duverger et al first demonstrated potential involvement of glycosylation in nuclear transport. A fluorescent-coupled sugar substituted bovine serum albumin (BSA), which was introduced either by electroporation or digitonin-permeabilisation of cells, was shown to reach the nucleus. Sugar residues enabling the transport were glucose, fructose and mannose while BSA coupled with galactoside or phosphomanoside remained in the cytosol (Duverger, Carpentier et al. 1993). Most significantly, sugar-dependent transport does not require nuclear localisation signals since N-ethylmaleimide, an alkylating agent that modifies nuclear localisation signals does not affect the transport. The transport is abolished at 4°C, suggesting that the transport is energy-dependent. Similar transport occurs with β-di-N-acetyle chitobiose-modified BSA, which shares a similar structure with GlcNAc coupled with serine or threonine residue (Duverger, Roche et al. 1996). In contrast to NLS-dependent transport, glycosylation-dependent transport of BSA is less efficient due to cytosolic retention and does not require importin. It is also cell cycle-dependent. Microinjection of fluorescence glycol-protein in Hela cells showed that the transport is more efficient during G1/S phase transition and S phase, whereas NLS-dependent transport does not show cell cycle dependency(Rondanino, Bousser et al. 2003).

Physiological observations concerning glycosylation and nuclear transport have been made with tau protein. Tau proteins are brain microtubule-associated proteins involved in polymerisation and stability of neuronal microtubules. Tau protein is phosphorylated and glycosylated reciprocally on the same serine/threonine residue (Lefebvre, Ferreira et al. 2003). Okadaic acid treatment increases the phosphorylated form and reduces nuclear localization, clearly demonstrating the importance of glycosylation in the transport process (Lefebvre, Ferreira et al. 2003). Tau proteins are hyperphosphorylated in diseased brain (Buee, Bussiere et al. 2000). They are devoid of O-GlcNAc residues and form aggregates in paired helical filaments in the cytosol. The balance between phosphorylation and glycosylation and the resulting changes in nuclear transport of protein, therefore, may have important implications for disease development

Although it is clear that O-linked modification of proteins is involved in nuclear transport, the exact mechanism mediating the process is unknown. Several groups suggest the involvement of glycosylation-binding protein or nuclear lectin in the process based on the observation that WGA inhibits nuclear transport by binding to GlcNAc moiety on nucleoporin (Finlay 1987), depletion of O-GlcNAc inhibits transport (Finlay 1990) and nucleoporin interacts with cytosolic factors that are required for transport. It has been suggested that transport could be mediated through cytosolonuclear lectins shuttling between cytosol and the nucleus. The recent isolation of nuclear and cytosolic N-acetyl glucosamine specific lectins from adult rat liver by affinity chromatography on immobilised GlcNAc (Lefebvre 2001) further substantiates this hypothesis.

A proteomic analysis utilising MALDI-TOF-MS and Protein Prospector/MS-FIT identify those lectins as heat shock proteins including a heat shock cognate-70 stress protein (hsc-70). The isolated lectins specifically recognise glycoprotein bearing O-GlcNAc residues present in both the cytosol and the nucleus of human resting lymphocytes. Lectinic activity of Hsp70 to GlcNAc labelled agarose beads is evident at both 37 and 42 °C. In HepG2 cells, its activity is modulated by stress and glucose availability. Surprisingly, its lectinic activity increases during glucose starvation. Although overall glycosylation of intracellular proteins decreases during starvation, immunoprecipitation and immunoblotting with glycosylation-specific antibody reveals that O-GlcNAc modified proteins such as beta-catenin are still detected with Hsp70. Increased affinity of Hsp70 toward beta catenin could account for the compensatory behaviour (Guinez 2004). The role of HSP 70 in nuclear transport is further confirmed by previous observation that depletion of HSP70 using ATP agarose chromatography of antibody lead to reduction in nuclear transport (Shi 1992). A similar observation is made with depletion of Hsp70 on the nuclear transport of the SV40 t antigen NLS-conjugated BSA (Okuno 1993). Though strong evidence link Hsp70 to nuclear translocation, the exact mechanism remains under intense study. Figure 1.21 demonstrates hypothetical interaction between HSP70 and O-GlcNAc modified protein.

Finally, glycosylation alters susceptibility of proteins to **proteasomal degradation**. Glycosylation prolongs the half-life of Sp-1, Myc, ER-β and eukaryotic initiation factor 2α. It links protein stability with nutritional state of the cells. In times of starvation, proteins are degraded rapidly, resulting in inactivity of downstream targets. Glycosylation alters protein stability through two separate mechanisms.

First, glycosylation competes with phosphorylation on residues that direct proteins to ubiquitination. On Myc, Thr 58 is phosphorylated by GSK3 upon Erk phosphorylation of

Ser62. GSK3 phosphorylation directs Myc for proteasomal degradation. Conversely glycosylation and modification of this residue, a hot spot found in lymphoma, increase Myc stability (Kamemura, Hayes et al. 2002). A second mechanism involves deceleration of proteasomal complex. Zhang et al assessed the *in vitro* peptidase cleavage of synthetic peptide substrates before and after O-GlcNAc modification of purified 26S, 20S proteasome and 19S sub complex. Glycosylation does not inhibit 20S or 26S activity but it diminishes ATPase activity of 19S sub complex (Zhang, Su et al. 2003). Still, deletion of OGT in mouse embryonic fibroblasts has little effect on overall rate of protein turn over *in vivo*, suggesting a complex substrate specific role in the control of protein degradation by glycosylation (O'Donnell, Zachara et al. 2004) or functional redundancy.

1.2.5.3.5.2 Control of metabolism

The hexosamine biosynthesis pathway is a cellular sensor of energy availability. The pathway acts as a negative feedback response for excess nutrient availability. It affects a range of cellular processes. Firstly, hexosamine pathway flux controls the rate of ATP production. At the cellular level ATP production is kept within a tight range. Prolonged activation of nutrient sensing pathways signals energy overflows. To limit marked excursion in the rate of cellular respiration, prolonged increases in hexosamine flux causes reduced efficacy in ATP production through reduced expression of mitochondrial genes involved in oxidative phosphorylation (Obici, Wang et al. 2002). These changes in gene expression are accompanied by a marked decrease in whole body energy expenditure. Secondly, the hexosamine flux controls appetite. Administration of glucosamine rapidly induces expression of leptin in muscle and fat in rodent (Wang, Liu et al. 1998) and humans (Considine, Cooksey et al. 2000) at the level of transcription (Zhang, Klenk et al. 2002), while inhibition of glucosamine production reduces glucose-stimulated leptin production from human adipose tissue (Considine, Cooksey et al. 2000). Thirdly, flux through the hexosamine pathway is a mechanism by which cells sense ambient glucose level. When glucose flux is excessive, this pathway transmits messages which downregulate glucose uptake into the cells. Ciaraldi et al showed that chronic treatment with glucosamine reduces glucose transport in skeletal muscle cell at a posttranslational level of GLUTs (Ciaraldi, Carter et al. 1999). Finally, the hexosamine pathway regulates the conversion of excess glucose into lipids by fat cells. In isolated rat adipocytes, glucosamine administration increases mRNA of lipogenic enzymes such as glycerol-3-phosphate dehydrogenase and fatty acid synthase (Rumberger, Wu et al. 2003).

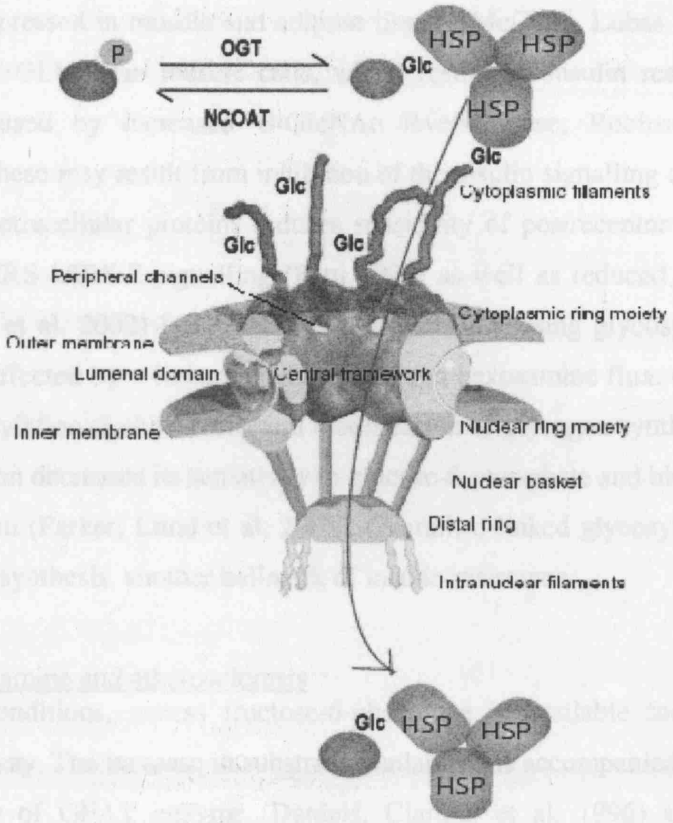


Figure1.21 Glycosylation and nuclear transport: nuclear Lectin bind to O-GlcNAc modified protein, bridging them with nuclear pore through O-GlcNAc residue on nucleoporin. Spatial proximity allows for an additional factor to enable translocation of proteins through pore complex into the nucleus. Phosphorylation and glycosylation could determine the balance of this transport, phosphorylation being a modification that retains proteins in the cytosol.

By controlling the sensitivity of proteins along signaling cascade to hormonal signals, glycosylation enables direct interaction between nutritional state of the cells and cellular response to extracellular stimuli (Wells, Vosseller et al. 2001). The state and the stoichiometry of O-GlcNAc, which is based on concentration of UDP-GlcNAc and nutritional state of the cells, can determine the magnitude of cellular response to extracellular stimuli by controlling the extent of phosphorylation and protein interaction.

1.2.5.3.5.3 Hexosamine and insulin resistance

Under physiological circumstances, hexosamine flux facilitates a negative feedback response that controls gene expression in response to nutritional status. However, prolonged increases in flux through this pathway can lead to eventual development of insulin resistance. Marshall et al first demonstrated the involvement of hexosamine flux in insulin resistance by using GFAT inhibitor azaserine to inhibit hyperglycaemia-induced insulin resistance (Marshall, Bacote et al. 1991). Since then more evidence from in vivo and in vitro models confirm the importance of hexosamine flux in insulin resistance. Insulin resistance is observed when

OGT has been expressed in muscle and adipose tissue (McClain, Lubas et al. 2002) while overexpression of GLUT1 in muscle cells, which results in insulin resistance, has been shown to be caused by increased O-GlcNAc levels (Buse, Robinson et al. 2002). Mechanistically, these may result from inhibition of the insulin signalling cascade. Increased glycosylation of intracellular proteins reduces sensitivity of postreceptor insulin signalling events. Reduced IRS-1/IRS-2 signalling (Patti 1999) as well as reduced PKB/Akt activity (Vosseller, Wells et al. 2002) has been observed with increasing glycosylation. Glycogen synthesis is also affected by o-linked glycosylation and hexosamine flux. GSK3β is a target of o-linked glycosylation (Lubas 2000) and modification of glycogen synthase enzyme of o-linked glycosylation decreases its sensitivity to glucose-6-phosphate and blocks phosphatase-mediated activation (Parker, Lund et al. 2003). Overall, o-linked glycosylation is linked to reduced glycogen synthesis, another hallmark of insulin resistance.

1.2.5.3.5.4: Hexosamine and atherosclerosis

Under diabetic conditions, excess fructose-6-phosphate is available for the hexosamine biosynthesis pathway. The increase in substrate availability is accompanied by an augmented level and activity of GFAT enzyme (Daniels, Ciaraldi et al. 1996) and OGT enzyme (Akimoto, Kreppel et al. 2001). Excess flux through hexosamine pathway mediates many proatherogenic effects of hyperglycaemia. Primarily, stimulation of Sp1 activity through O linked glycation and PKC-βI dependent mechanisms (Goldberg, Whiteside et al. 2002) leads to increased activation of PAI-1 promotor through Sp1 sites (Du 2000). This overexpression is blocked by azaserine, an inhibitor of GFAT (Goldberg 2000). Another contributing mechanism by hexosamine flux is the induction of proinflammatory cytokines TGF-α and TGF-β1 by hyperglycemia, which are also blocked by azaserine (Kolm-Litty, Sauer et al. 1998). Additionally, glucosamine is found to enhance PDGF-induced mitogenesis of smooth muscle cells, possibly contributing to accelerated lesion development (Federici, Menghini et al. 2002). Finally, excess flux through the hexosamine pathways reduces the expression of atheroprotective nitric oxide. With high glucose, the phosphorylation of Ser 1177 of eNOS enzyme is replaced with inhibitory glycosylation on the same residue, causing a reduction in nitric oxide generation (Du 2001). Impairment of eNOS activation is worsened by O-linked glycation-mediated inhibition of IRS, PI3 kinase and PKB (Federici, Menghini et al. 2002).

1.3 Transcription factors and lipid homeostasis

Mechanisms linking decreased insulin sensitivity with the development of coronary artery risk factors may occur at the molecular or gene regulatory level. Transcription factors are

prime candidates because they regulate the expression of many genes and are targets of different intracellular signalling cascades, coupling extracellular signals to gene expression. As far as atherosclerosis is concerned, PPAR and SREBP are two transcription factors that attracted much interest because of their established role in lipid regulation. Brief outlines of both factors are described below.

1.3.1 PPAR

PPARs are members of the nuclear receptor family that function as lipid sensors. Three different genes encode the α β/δ γ isoforms. Changes in lipid concentration directly alter expression of proteins involved in lipid uptake, synthesis, transport, storage, degradation and elimination.

1.3.1.1 PPAR protein structure and ligand

PPARs contain DNA and ligand binding domains, well conserved in the nuclear receptor family. The DNA binding domain consists of two zinc finger binding domain that recognise the peroxisome proliferator response element (PPRE), consisting of two hexanucleotide repeat with the consensus sequence AGGTCA separated by a single nucleotide spacer. The C-terminal ligand binding domain is relatively large compared with other nuclear receptors, possibly allowing PPAR to react with a wide range of ligands. Various direct binding studies identify fatty acid and eicosanoid derivatives as natural ligand of PPAR. PPAR-γ prefers polyunsaturated fatty acids, including linoleic acid, arachidonic acid, eicosapentaenoic acid (Kliewer, Sundseth et al. 1997) and 15–deoxy-12,14-prostaglandin J2(Nagy 1998). PPAR-α prefers oxidised fatty acids, conjugated fatty acid and fatty acid-derived eicosanoids(Willson, Brown et al. 2000). Thiazolidinediones (TZD) such as rosiglitazone, pioglitazone, englitazone and ciglitazone are well-studied synthetic ligands for PPAR - γ(Lehmann, Moore et al. 1995).

1.3.1.2 Mechanism of action α

PPARs form heterodimers with retinoid x receptor (RxR). In the absence of ligand, PPAR/RxR heterodimer can bind to specific response elements in target genes and actively repress transcription through interaction with corepressor complexes that contains the nuclear receptor corepressors NCoR or SMRT(Krogsdam, Nielsen et al. 2002; Shi, Hon et al. 2002; Hu, Li et al. 2003). In the presence of ligand, PPAR/RxR heterodimer activate transcription through ligand-mediated allosteric changes that lead to the recruitment of coactivator proteins(Rosenfeld and Glass 2001; McKenna and O'Malley 2002). Several co activators such as CBP/p300 and steroid receptor coactivator-1 possess histone acetylase activity that can remodel chromatin structure(Zhu, Qi et al. 1996). The second group with

PPAR binding protein form a bridge between the nuclear receptor and transcription initiation machinery (Zhu, Qi et al. 1997). The third group such as PGC-1 is not yet well understood. PPAR is modified posttranslationally through phosphorylation and dephosphorylation. Insulin-activated phosphorylation increases PPAR-γ activity while beta adrenergic signalling decreases PPAR-γ activity (Zhang, Berger et al. 1996).

1.3.1.3 Intracellular function of PPAR

PPAR-α and PPAR-γ play prominent roles in lipid and glucose homeostasis in adipocytes and in macrophages. The ability of TZD compounds to improve insulin resistance points to the importance of PPAR-γ in glucose homeostasis. TZDs partly achieve this through inhibition of TNF-α secretion from adipose tissue (Peraldi, Xu et al. 1997) and an induction of cbl and IRS-2 expression (Tamori, Masugi et al. 2002). By promoting adipocyte differentiation and free fatty acid uptake, TZD may indirectly improve insulin resistance by reducing circulating free fatty acids. Tissue-specific deletion of PPAR-γ in adipose tissue points to the role of PPAR-γ in regulating insulin resistance in fat, muscle and liver (Gavrilova, Haluzik et al. 2003; He, Barak et al. 2003; Hevener, He et al. 2003). Other studies uncover the role of PPAR-γ in the induction of GLUT4 expression (Wu, Xie et al. 1998), release of fatty acid from chylomicrons and VLDL(Lefebvre, Peinado-Onsurbe et al. 1997), upregulation of genes involved in fatty acid transport, synthesis and esterification (Brun, Tontonoz et al. 1996). In adipocytes, PPAR-γ regulates many genes involved in lipid metabolism including ap2 (Tontonoz, Hu et al. 1994),acyl CoA synthase and LPL (Bogacka, Xie et al. 2004). It regulates UCP proteins, controlling energy expenditure (Kelly, Vicario et al. 1998). PPAR-α knockout mice reveal the role of PPAR-α in fatty acid oxidation. Upon fasting PPAR-α activity increases in order to increase gene expression of enzymes that degrade fatty acids and produce ketone body to serve as fuel (Leone, Weinheimer et al. 1999). PPAR-α activates fatty acid transport protein (Motojima, Passilly et al. 1998), long chain acyl Co A synthase gene (Schoonjans, Watanabe et al. 1995) and HMG CoA synthase (Rodriguez, Gil-Gomez et al. 1994) for the purpose. PPAR-α activation also increases Apo AI (Vu-Dac, Chopin-Delannoy et al. 1998) and reduces Apo CIII (Clavey, Copin et al. 1999). Together these effects increase HDL level and reduced circulating triglyceride.

1.3.1.4 PPAR and lipid metabolism in macrophages

PPAR-γ is involved in the uptake, intracellular metabolism and efflux of lipids in macrophages. First of all, it is involved in upregulation of CD36 receptor by oxLDL (Feng, Han et al. 2000). Exposure of J774.2 macrophages to oxLDL results in a marked induction of CD36 mRNA and protein (Han 1997) through the activation of PPAR by oxLDL (Nagy

1998; Tontonoz 1998). Components of the oxLDL particle, 9-hydroxyoctadecadienoic acid and 13-hydroxyoctadecadienoic acid transcriptionally activate PPAR-γ. Other PPAR-γ ligands such as 15-deoxy-12,14-prostaglandin J2 and thiazolidinediones also increase CD 36 expression (Feng, Han et al. 2000). PPAR-γ is vital for basal regulation of CD36 (Chawla 2001). The injection of PPAR-γ deficient embryonic stem cells into wild-type blastocysts causes reduction in CD36 expression and PPAR agonists fail to induce CD36 expression. This suggests the possibility that macrophage expression of CD36 in response to oxysterol and PPAR-γ agonists may create a cycle in which oxLDL drives its own uptake leading to accelerated foam cell formation. However, PPAR-γ ligands do not promote foam cell formation after acetylated LDL loading in human macrophages (Chinetti, Lestavel et al. 2001). Recent findings also showed that in the context of the pre-existing metabolic syndrome found in ob/ob mice, TZD treatment reduces CD36 protein levels through a posttranslational effect (Liang, Han et al. 2004). The increase in CD36 expression may be compensated by inhibitory effect of PPAR-γ ligands on SR-A expression (Ricote 1998) and its stimulatory effect on cholesterol efflux.

Next, TZD has been found to increase to apolipoprotein AI-dependent efflux of cholesterol from both murine and human macrophages through upregulation of ABCA-1 (Chawla, Boisvert et al. 2001). Ligand activation of PPAR-γ leads to primary induction of oxysterol receptor LxR-α, a direct transcriptional target of PPAR-γ/RxR heterodimers, followed by LxR/RxR activation of ABCA promoter (Chinetti, Lestavel et al. 2001). The cytochrome P450 Cyp27, which catalyses production of LxR agonist 27-hydroxycholesterol, is another target of PPAR-γ. Its activation contributes to the integration of PPAR to LxR control of efflux pathway by allowing PPAR to generate ligands for LxR (Szanto, Benko et al. 2004). Alternatively, recent experiments (Li, Binder et al. 2004) demonstrated that PPAR-γ agonists can inhibit foam cell formation within the peritoneal cavities of hypercholesterolemic LDLR-/- mice without affecting expression of LxR-α or ABCA1. Analysis shows that the PPAR agonist rosiglitazone increases ACAT1 activity through posttranslational effect while the increase in HDL-dependent efflux is mediated through the up regulation of ABCG1 .

In summary, by simultaneously inducing oxLDL intake, cholesterol esterification and cholesterol efflux through CD36, ACAT activation and ABCA-1/ABCG up regulation respectively, PPAR-γ accelerates the removal of atherogenic oxLDL from vessel wall and its removal through HDL-mediated transport to the liver.

The atheroprotective effect of PPAR-γ has been confirmed through an experiment involving bone marrow transplantation from either wild type or PPAR-γ null mice into LDL receptor

null mice. Bone marrow from PPAR-γ null mice causes marked increase in atherosclerotic lesion area, suggesting that PPAR-γ and its transcriptional target has highly atheroprotective property. Moreover, Rosiglitazone and GW7845 inhibit development of atherosclerosis in LDL-receptor deficient male mice despite increased expression of CD 36 in the arterial wall (Li and C.K. 2000; Collins and G. 2001). A similar result has been obtained from ApoE knock out mice fed a high fat diet (Chen, Tamura et al. 2001.). In humans, two small scale clinical trials exhibit reduced carotid intimal thickening in diabetic patients treated with TZD (Minamikawa, Tanaka et al. 1998; Koshiyama, Shimono et al. 2001). Anti-inflammatory effects of PPAR-γ agonists could contribute to its antiatherogenic property. Treatment of human macrophages with natural and synthetic PPAR-γ ligands suppress inflammatory mediators such as inducible nitric oxide synthase, tumor necrosis factor-α, interleukin 6 and interleukin 1β (Ricote 1998). However, these effects are not mediated through PPAR-γ as the macrophage inflammatory responses driven by lipopolysaccharide derived from PPAR-γ null embryonic stem cells remain inhibited by TZD and 15d-PGJ2. 15-deoxy-12,14 prostaglandin J2, a natural ligand of PPAR -γ, are able to prevent the activation of NF-kB by directly inhibiting IkB kinase(Rossi, Kapahi et al. 2000; Straus, Pascual et al. 2000).

PPAR-α agonists also exert a similar effect on ABCA expression and inhibition of ACAT activity in THP-1 cells (Chinetti, Lestavel et al. 2001; Chinetti, Lestavel et al. 2003) but studies in animal models show conflicting results. PPAR-α and Apo E double knock out mice exhibit less atherosclerosis than control mice (Tordjman, Bernal-Mizrachi et al. 2001), suggesting a proatherogenic role of PPAR-α. Treatment of Apo E knockout mice with PPAR-α-specific ciprofibrate worsened diet-induced hyperlipidaemia and increased atherosclerosis (Fu, Kashireddy et al. 2003). On the other hand, use of a potent agonist such as GW7647 improves lesion size in LDLR knockout mice (Li, Binder et al. 2004). GW7647 does not affect ABCA expression, its beneficial effect on foam cell formation actually occurs independently of cholesterol esterification or cholesterol efflux. The beneficial effect is abolished once bone marrow from LxR-α knock out mice is transferred into LDLR knockout mice prior to treatment with PPAR-α agonist, suggesting an LxR dependent but ABCA independent effect. This effect could be dependent on ApoE, hence the lack of improvement in ApoE knock out mice.

1.3.2 Sterol regulatory Element Binding Proteins (SREBPs)

1.3.2.1 Function

SREBPs have now been established as global regulators of lipid synthesis. Their function has been studied extensively in liver, adipose tissue and muscle. SREBP regulates the

expression of many lipogenic enzymes involved in energy storage through synthesis of fatty acid and triglycerides (Hillgartner, Salati et al. 1995; Horton, Shah et al. 2003). A series of overexpression and knockout analyses demonstrate the crucial role of SREBP-1c in the control of hepatic lipogenesis (Shimano, Horton et al. 1996; Shimano, Horton et al. 1997; Shimano, Yahagi et al. 1999) and the control of glucokinase induction by insulin(Foretz, Pacot et al. 1999). Similar experiments demonstrate the role of SREBP-1a in the control of both cholesterol synthetic genes and fatty acid synthesis genes in the liver (Shimano, Horton et al. 1996; Horton, Shah et al. 2003), while hepatic overexpression of SREBP-2 causes induction of cholesterol synthetic genes and moderate induction of fatty acid synthetic genes(Horton, Shimomura et al. 1998). Regulation of a multitude of enzymes through a single mediator such as SREBP allows for coordinated regulation in response to cellular nutritional status.

1.3.2.2 SREBP and metabolic disease

An increasing number of genetic studies implicate SREBP in the development of diabetes, obesity and atherosclerosis. The SREBF locus has been linked to type 2 diabetes (Demenais, Kanninen et al. 2003) and SNPs of SREBF-1 gene have been linked to obesity, diabetes (Eberle, Clement et al. 2004) as well as an atherogenic lipid profile in men at high cardiovascular risk (Salek, Lutucuta et al. 2002). In adipose tissue and muscle of type 2 diabetic patients, mRNA of SREBP-1c decreases in comparison to lean control (Ducluzeau, Perretti et al. 2001; Oberkofler, Fukushima et al. 2002; Sewter, Berger et al. 2002). Microarray studies also show a reduction in SREBP level in adipose tissue of ob/ob mice as well as in obese mice (Nadler, Stoehr et al. 2000). SREBP-1c mRNA is lower in muscle of diabetic compared to lean control while the level increases in fatty liver of obese, insulin resistant and hyperinsulinaemic ob/ob mice (Shimomura, Matsuda et al. 2000). In contrast to a reduction in the liver, SREBP-1 levels in renal cell increases in kidney of streptozotocin diabetic mice, causing increased lipid deposit leading to glomerulosclerosis (Sun, Halaihel et al. 2002). Together, evidence shows that dysregulation of lipogenesis and increased lipid in non-adipose tissues in diabetes may be caused by dysregulation of SREBP expression.

1.3.2.3 Regulation of SREBP maturation

SREBP is a bHLH-zip domain containing transcription factor that binds to the sterol regulatory element (TCACnCCAC) and E box element (CannTG). Structurally, there are three essential domains on SREBP: the N-terminal domain of approximately 500 amino acids containing the transactivation domain and the bHLH-zip region , the transmembrane domain containing a short loop of approximately 80 amino acids and the C-terminal domain of approximately 590 amino acids(Sato, Yang et al. 1994).

There are three SREBP isoforms designated SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are generated from the same gene by recruitment of two distinct promoters and different first exons. Exon 1 of SREBP -1a contains 29 amino acids while exon 1 of SREBP-1c contains only 9 amino acids. Both are connected to exon 2 by alternative splicing. When overexpressed, all isoforms can activate SRE containing promoters (Pai, Guryev et al. 1998). Mice overexpressing active SREBP-1c in the liver have a 25-fold higher rate of hepatic lipid synthesis while those overxpressing SREBP-2 show 25-fold increase in hepatic cholesterol synthesis (Horton, Goldstein et al. 2002), demonstrating the role of SREBP-1 in lipid synthesis and SREBP-2 in cholesterol synthesis.

SREBPs are synthesised as precursors and inserted into the endoplasmic reticulum membrane where they are anchored through the membrane spanning domains with both amino and carboxylic domain facing the cytosolic face of the membrane. In sterol-deficient cells, proteolytic cleavage by SREBP-specific proteases (S1P and S2P) occurs in the Golgi body, thereby releasing the N-terminal mature form from the membrane, enabling them to enter the nucleus where they bind to SRE or E box sequence.

Maturation of SREBP is controlled at the level of ER to Golgi transport. In the ER, SREBP is found in a complex with SCAP protein (Sakai, Duncan et al. 1996) through the cytoplasmic carboxy terminus of SCAP and carboxy terminal domain of SREBP(Sakai, Nohturfft et al. 1997). In sterol-depleted cells, SCAP-SREBP complex enters budding ER vesicles and travels to the golgi (Nohturfft, Yabe et al. 2000). On the other hand, the complex is retained in the ER when intracellular cholesterol level is high through the interaction of SCAP with INSIG protein through the N terminal sterol sensing domain of SCAP (Yang, Espenshade et al. 2002). Mutation in this domain causes a gain of SREBP function, with constitutive transport to the golgi that is resistant to high levels of intracellular cholesterol (Nohturfft, Brown et al. 1998; Nohturfft, Brown et al. 1998). Overexpression of the sterol sensing domain of SCAP alone reproduces a similar effect through over saturation of INSIG (Yang, Goldstein et al. 2000).

A reconstitution experiment in insect cells demonstrates that SCAP and INSIG are the two most basic entities for sterol-regulated transport of SREBP (Dobrosotskaya, Goldstein et al. 2003). In a sterol-rich environment, the interaction between INSIG and SCAP is enhanced (Yang, Goldstein et al. 2000) through cholesterol-mediated conformational change of cytoplasmic loop of SCAP (Brown, Sun et al. 2002). In effect, binding of INSIG to SCAP in the presence of cholesterol leads to ER retention by inhibiting Sar1-dependent binding of the

COPII proteins Sec 23/24 to SCAP, inhibiting access of SCAP-SREBP complex to COPII coated vesicle in transitional ER (Espenshade, Li et al. 2002). 25-hydroxy-cholesterol, C18 fatty acids and PUFA (Yahagi, Shimano et al. 1999) are strong inhibitors of SREBP processing but the level of inhibition differs between SREBP-1 and SREBP-2. SREBP-1 cleavage is less sensitive to 25-hydroxysterol inhibition, although the level decreases and is more sensitive to inhibition by oleate. Potency of inhibition by fatty acid increases with increasing chain length and degree of unsaturation with the reduction occurring at both mRNA level and during cleavage process of SREBP-1 (Hannah, Ou et al. 2001). Furthermore, cleavage of SREBP-1c is more sensitive to nutritional status and insulin than sterol (Hegarty, Bobard et al. 2005). While expression of SREBP-1c is responsive to LxR and insulin, the cleavage of SREBP-1c essentially requires insulin. SREBP-1c induced by LxR agonist does not generate a mature form but a short pulse of insulin of 20-45 minutes generates cleavage from existing precursor forms. Insulin per se is able to rapidly modulate the nuclear SREBP-1 concentration in the nucleus independent of any effect on SREBP-1c transcription (Hegarty, Bobard et al. 2005). To date the mechanism by which insulin controls SREBP-1 processing has not been elucidated, but the increase in Insig-1 mRNA concomitantly with a decrease in Insig-2 mRNA in response to insulin may contribute to the regulation (Yabe, Komuro et al. 2003). There is a possibility that Insig-2 is a specific Insig for SREBP-1c, hence depression by insulin causes an increase in nuclear SREBP-1c level.

On the other hand, SREBP-2 is more sensitive to sterol inhibition (Hasty, Shimano et al. 2000). An experiment in vivo based in hamster liver shows that hepatic SREBP-1 mature form decreases in response to sterol depletion and cholesterol synthesis inhibitor while level of SREBP-2 increases (Sheng, Otani et al. 1995). Nonetheless, a combination of 25-hydroxy cholesterol with oleate potentiates the cleavage inhibition of both SREBP-1 and SREBP-2 (Thewke, Panini et al. 1998; Hannah, Ou et al. 2001).

Interestingly the cleavage process differs between strongly lipogenic tissues such as adipocytes and tissues evolved for other purposes such as islet cells. Unlike liver cells, the mature form of SREBP-1 is not detected in islet cells after 6 hours treatment with 30mM glucose and insulin, but appears after 48 hours treatment in glucose or insulin (Wang, Maechler et al. 2003). The increase in SREBP-1 mature form after prolonged exposure may lead to renal lipid accumulation and diabetic nephropathy (Sun, Halaihel et al. 2002).

Once transported to the Golgi, SREBP is cleaved at the loop region and the transmembrane region by site 1 and site 2 proteases to liberate the N terminal region of SREBP (Sakai, Duncan et al. 1996). Transcriptionally active portions of SREBP then enter the nucleus

through nuclear pore complex. Extensive analysis has been carried out with SREBP-2. The bHLH-zip motif of the SREBP-2 contains a novel type of nuclear localisation signal that interacts directly with importin beta. The transport occurs through importin beta-mediated shuttling and Ran-dependent dissociation as shown by transport assay in vitro (Nagoshi, Imamoto et al. 1999). Dimerisation of SREBP is a prerequisite for the import (Nagoshi and Yoneda 2001).

1.3.2.4 Coregulatory factors

SREBP itself is a weak transcription factor. Coregulatory transcription factors which bind to nearby DNA sequences are required to potentiate the effects of SREBP. Known coregulators are Sp1 for LDL receptor gene (Sanchez, Yieh et al. 1995), NF-Y for farnesyl diphosphate synthase gene (Ericsson, Jackson et al. 1996), CREB for hydroxymethyl glutaryl CoA reductase (Bennett and Osborne 2000) and nuclear thyroid hormone receptor and Retinoid x receptor for Acetyl CoA Carboxylase--α in hepatocytes. The latter interaction increases the conversion of glucose to triacylglycerol (Yin, Zhang et al. 2002). Increased acetylation of histone H3 has also been detected upon SREBP activation as demonstrated through the chromatin immunoprecipitation technique (Bennett and Osborne 2000).

1.3.2.5 Interaction with inhibitory factors

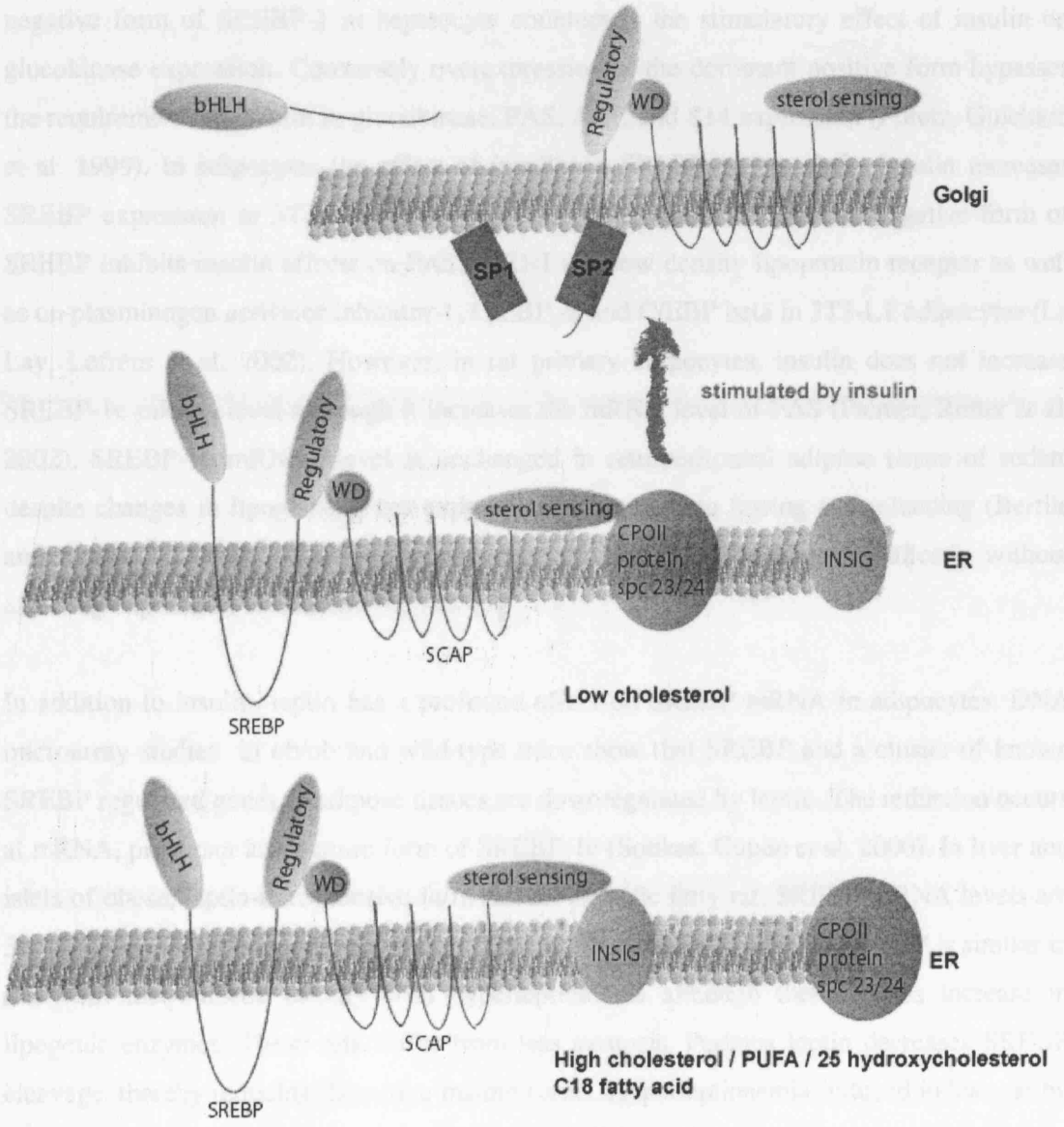
The transcriptional activity of SREBP is further modulated by direct interaction with inhibitory proteins. SREBP-1c interacts with Twist2, a bHLH-containing protein, in adipocytes and hepatocytes. Overexpression of Twist 2 represses the transcriptional activity of SREBP-1c by reducing its binding to target sequences. This inhibition is enhanced upon co-expression with HDAC and relieved with HDAC inhibitor, suggesting that HDAC is involved with inhibitory effect of Twist2(Lee, Lee et al. 2003). In adipocytes, SREBP has been found in complex with Id2. The interaction inhibits the formation of SREBP-DNA complex, reducing the activity of SREBP target promotor. The interaction has been implicated in the inhibitory effect of cAMP and other antilipogenic agents on SREBP target gene such as FAS as Id2 level increases with rising level of cAMP(Moldes 1999). Id2 is discussed in more detail in the next section 1.3.3.

1.3.2.6 Regulation of SREBP expression

The expression of SREBP-1c is tightly linked to carbohydrate metabolism. SREBP-1c levels are depressed during fasting but increases markedly after refeeding with high carbohydrate diet in mouse liver, white adipose tissue and skeletal muscle (Horton, Shimomura et al. 1998; Kim, Sarraf et al. 1998; Bizeau, MacLean et al. 2003; Commerford, Peng et al. 2004; Gosmain, Dif et al. 2005). The effect is much less for SREBP-1a or SREBP-2 mRNA.

Transcription of SREBP is under feed forward control mediated by sterol response element present in the promoters of both SREBP-1 and SREBP-2 (Sato, Inoue et al. 1996). Binding of SREBP active fraction to SRE 3 site is responsible for the basal promoter activity. Further activation is conferred through NF-Y site. This positive regulation could be responsible for augmented effect observed after refeeding.

Figure 1.22 Processing of SREBP



SREBP-1c expression is also under the control of insulin. It is upregulated by insulin *in vivo* and in primary hepatocyte culture (Foretz, Guichard et al. 1999; Azzout-Marniche, Becard et al. 2000), isolated adipocytes (Kim, Sarraf et al. 1998), muscles (Guillet-Deniau, Mieulet et al. 2002) and streptozotocin-diabetic rat (Shimomura, Bashmakov et al. 1999). Induction of SREBP promoter transfected into rat hepatocyte occurs regardless of whether 5mM or 20mM glucose is in the treatment medium (Deng, Cagen et al. 2002). Similar induction

occurs in adipose tissue and muscle of healthy human patients participating in 3 hours hyperinsulinaemic-euglycaemic clamp (Ducluzeau, Perretti et al. 2001). SREBP has been implicated as a mediator of insulin effect on hepatic lipogenesis since the increase in level of SREBP occurs in parallel with induction of mRNA of its target genes such as fatty acid synthase (Foretz, Guichard et al. 1999). In SREBP-1c knockout mice, there is marked decrease in insulin-induced stimulation of hepatic lipogenic gene expression that normally accompany feeding and refeeding (Shimano, Yahagi et al. 1999). Expression of the dominant negative form of SREBP-1 in hepatocyte counteracts the stimulatory effect of insulin on glucokinase expression. Conversely overexpression of the dominant positive form bypasses the requirement for insulin in glucokinase, FAS, ACC and S14 expression (Foretz, Guichard et al. 1999). In adipocytes the effect of insulin on SREBP is less clear. Insulin increases SREBP expression in 3T3-L1 adipocytes (Kim 1998) and the dominant negative form of SREBP inhibits insulin effects on FAS, SCD-1 and low density lipoprotein receptor as well as on plasminogen activator inhibitor-1, C/EBP -α and C/EBP beta in 3T3-L1 adipocytes (Le Lay, Lefrere et al. 2002). However, in rat primary adipocytes, insulin does not increase SREBP-1c mRNA level although it increases the mRNA level of FAS (Palmer, Rutter et al. 2002). SREBP-1c mRNA level is unchanged in retroperitoneal adipose tissue of rodent despite changes in lipogenic genes expression in response to fasting and refeeding (Bertile and Raclot 2004). In these cells insulin may affect the cleavage process directly without affecting expression of SREBP-1c.

In addition to insulin, leptin has a profound effect on SREBP mRNA in adipocytes. DNA microarray studies in ob/ob and wild-type mice show that SREBP and a cluster of known SREBP regulated genes in adipose tissues are downregulated by leptin. The reduction occurs at mRNA, precursor and mature form of SREBP-1c (Soukas, Cohen et al. 2000). In liver and islets of obese, leptin-unresponsive fa/fa Zucker diabetic fatty rat, SREBP mRNA levels are 3-4 times higher than in the lean control. The increase in mRNA level of SREBP is similar in rats with diet-induced obesity with hyperleptinaemia although there is less increase in lipogenic enzymes. These rats suffer from less steatosis. Perhaps leptin decreases SREBP cleavage, thereby reducing the active mature forms. Hyperleptinaemia-induced in lean rat by adenovirus induction also causes reduction in SREBP mRNA level; therefore leptin may affect both transcription and processing of SREBP (Kakuma, Lee et al. 2002). These results suggest that increased lipogenesis and lipotoxicity in response to leptin resistance could be a result of SREBP overexpression.

Adenovirus-mediated transfection of dominant negative SREBP into rat hepatocyte implicates SREBP-1 as a mediator of glucose effects on hepatic lipogenic genes (Foretz,

Guichard et al. 1999). Experiments carried out in the H2-35 hepatic cell line shows that glucose increases the level of SREBP-1c mRNA, mature form as well as precursor form of SREBP-1 starting from 12 hours and this is maintained for up to 48 hours (Hasty, Shimano et al. 2000). Similar induction occurs in liver of streptozotocin-induced diabetic mice (Matsuzaka, Shimano et al. 2004). A more acute effect has been observed in rat skeletal muscle (Guillet-Deniau, Pichard et al. 2004). However, the glucose effect does not occur in primary rat hepatocytes. The reason underlying this discrepancy is unclear and could be species specific (Deng, Cagen et al. 2002).

Expression of SREBP-1 also comes under the control of dietary lipids. A cholesterol-rich diet significantly increases SREBP-1c expression in mouse liver without affecting the SREBP-1a or SREBP-2 (Repa, Liang et al. 2000). The increase in SREBP-1c mRNA in response to cholesterol is mediated through LxR/RxR nuclear hormone receptors that are bound to and activated by oxysterol (Repa, Liang et al. 2000). The effect is mimicked by synthetic ligands of LxR (Schultz, Tu et al. 2000) and in mice lacking LxR genes, basal expression of hepatic SREBP mRNA is significantly reduced in parallel with mRNA level of lipogenic genes (Repa, Liang et al. 2000) . On the other hand PUFA-rich diet represses the transcription of lipogenic genes by suppressing lipogenic SREBP-1 expression. PUFA antagonises the activation of LxR by LxR endogenous ligands, resulting in the inhibition of hepatic SREBP expression (Ou, Tu et al. 2001). It also accelerates the rate of SREBP-1 mRNA turn over (Xu, Teran-Garcia et al. 2001). PUFA also inhibits SREBP processing, possibly by increasing the intracellular pool of cholesterol (Worgall, Sturley et al. 1998). Activation of SREBP-1 could be an important link between fatty acid synthesis and cholesterol ester production in the liver. One of the target genes of SREBP is Stearoyl CoA desaturase-1(Tabor, Kim et al. 1999), an enzyme catalysing the conversion of stearoyl-CoA into Oleoyl-CoA, an important substrate for cholesterol ester synthesis. Thus, induction of SREBP-1 under high cholesterol would promote esterification of free cholesterol into cholesterol ester by ACAT enzyme in the liver before its export into other tissues.

1.3.3 Inhibitor of DNA binding (Id) Proteins

1.3.3.1 Description of HLH factors

Id2 is a member of a family of transcription factors containing the helix-loop-helix (HLH) domain. There are over 250 representatives in organisms ranging from yeast to man and are one of the most versatile families of eukaryotic transcription factors. The bHLH family can be divided into subgroups on the basis of structure, expression pattern and DNA binding activity (Massari 2000). The main subgroups are the HLH (Helix-loop-helix) and HLH-LZ

(helix-loop-helix leucine zipper). Binding of these transcription factors to DNA is mediated via the basic region immediately adjacent to the HLH region and is dependent upon the formation of homo- or heterodimers of these transcription factors. Upon dimerisation, they recognize E box sequence (CAnnTG) on DNA, recruit co-factors and activate or repress transcription of many genes (Grandori 2000; Massari 2000). Permutations in pairing of these factors result in the fine tuning of gene expression.

HLH transcription factors can be subdivided into two classes. Class A proteins are ubiquitously expressed and are able to form homo- or heterodimers with class B or tissue-specific bHLH factors. Class B factors form homodimers poorly but readily form heterodimers with class A factors. Id proteins belong to class B. Ids lack the DNA binding basic region common to other HLH transcription factors. Members of the Id family have been named Id1, Id2, Id3 and Id4. All of them are similar in size (13-20 kDa). Id1 was first cloned by Benezra et al (Benezra 1990). Id3 was isolated as a mitogen-responsive early response gene (Christy 1991) and Id2, Id4 by homology cloning (Sun 1991; Riechmann 1994; Pagliuca 1995).

Table 1.2 : HLH proteins

Family	**Class**	**Protein**
HLH	A	E12, E47, E2-5, E2A
		E2-2, HEB,REB, HTF4
	B Myogenic	MyoD, Myf-5, MRF4, Myogenin
	B Neurogenic	Neurogenin, NeuroD1, NeuroD2, NeuroD3
		NSCL-1, NSCL-2
		Mash-1, Mash-2
		Hes-1, -2, -3, -5
	B Haemopoietic	SCL
		Tal-1, Tal-2
		Lyl-1
	B Cardiogenic	eHand, dHand
HLH without basic region		Id1-Id4
HLH-Lz	Max network	Myc, Mad 1-4, Mnt/Rox

	Adipocyte development	ADD-1/SREBP-1
	Muscle development	Twist
	Skeletal development	Scl-1

1.3.3.2 Association of Id2 with other transcription factors

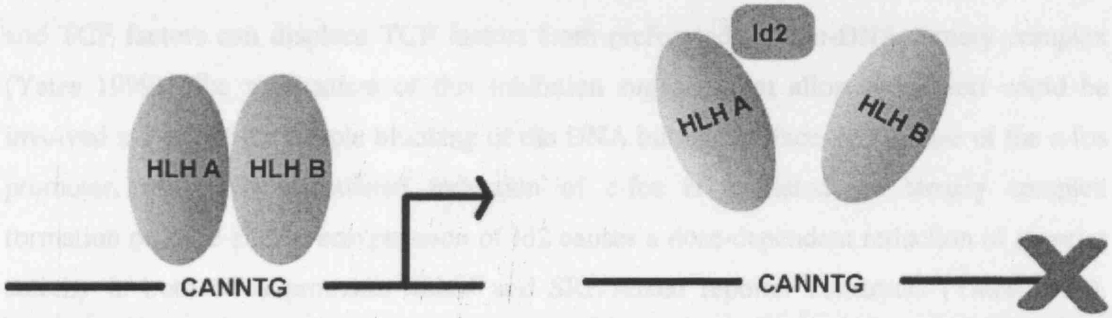
Members of the Id subfamily are able to form inactive heterodimers with other bHLH transcription factors via the helix-loop-helix motif. The HLH domain is comprised of two amphipathic α helices separated by an intervening loop. In the predicted Id3-HLH heterodimer, the individual HLH monomer packs to form a four α-helical bundle structure stabilised through electrostatic and polar interactions at the monomer interface (Wibley, Deed et al. 1996). The variable loop appears to be important for heterodimerisation and the heterodimers could be further stabilised by adopting a higher order multiple oligomeric states. Affinity selection experiments involving a screen of phage library identified important residues for strong association between HLH domains (Ciarapica 2003). The consensus sequence is shown in table 1.5. The presence of hydrophobic residues correlates to strong association with HLH domain, confirming the importance of hydrophobic core at the dimerisation interface (Goldfarb 1996). The preference for two loop residues Gln22 and Thr23 confirms the importance of the loop in HLH binding. Several charged residues at the interface appear to represent discontinuity points that are important for molecular recognition and prevent excessively strong interaction in order to allow physiological partner interchange (Ciarapica 2003).

Id proteins selectively bind to and inhibit the function of a distinct set of bHLH proteins. Id1 and Id2 bind and inhibit E2A, E47 and E2B.m3 (Sun 1991) E12, E2-2 and HEB (Langlands 1997) but not that of other sets including TFE3, USF and AP4. Id2 forms homodimers only poorly (Sun 1991). Generally HLH transcription factors of class A appear to associate strongly with Ids while class B tends to heterodimerise poorly. In most cell types, Id proteins probably modulate the function of class B HLH through sequestration of their class A and class E binding partners (Benezra 1990; Sun 1991; Littlewood 1995). By forming transcriptionally inactive heterodimer with E proteins, Id2 prevents them from forming functional dimers with tissue specific bHLH proteins (Norton 2000).

Table 1.3 Amino acid residues along HLH chain

Id2	strong binders	I V L	V L Q	L F	D K E N	T N A	G K	M V	C G S	Q	T	L I	T I	K I V	V Y	Q P
	weak binders	I V L	V L	L	D N K	E D A K	E K G	M V L	C G	Q	T S	L I V	T I	K I E	V Y D F	Q P

Figure 1.23 Inhibition of bHLH factors DNA binding by Id2



In some circumstances, Id2 can form heterodimers with tissue-specific class B bHLH factor such as HES-1 in neuronal cell (Jogi 2002) and MyoD, Myf-5 myogenic factors in muscle cells (Langlands 1997). In neuronal cells, Id2 forms transcriptionally inactive complex with Hes-1 and E2-2. When it is not bounded to Id2, Hes-1 forms an activator complex with HASH-1. Id2 cannot bind to HASH-1 or dHand (Sasai 1992). Binding of the activator complex HASH-1/E2-2 to DNA is reduced in the presence of Id2 by Id2 sequestration of E2-2. Hes-1 in turn sequesters Id2, allowing the formation of an active HASH-1/E2-2 complex. Consequently, the balance between HES-1 and Id2 levels regulates neuronal crest formation (Nakamura 2000). In addition to the inhibition of DNA binding, Id2 also inhibits gene expression by sequestering nuclear bHLH proteins to the cytoplasm. Id2 binds to OLIG factors, which are required for the generation of oligodendrocyte lineage commitment, in response to BMP. Following the exposure, OLIG changes from a predominantly nuclear localisation to a predominantly cytoplasmic localisation. Id2 concentration in the cytoplasm must reach a plateau before they sequester their partners away from the nucleus (Samanta and Kessler 2004). The binding partners of Id2 are summarised in table 1.6. In addition to bHLH factors, Id2 can heterodimerise with non-HLH factors such as hypophosphorylated pRb and ternary complex factor.

Ternary complex factors (TCFs) comprise a subfamily of ETS domain transcription factor. These proteins form ternary complexes with the serum response factor (SRF) and serum

response element (SRE) found in immediate early genes such as c-fos (Treisman 1994). Each protein shares four conserved regions, the ETS domain near the N terminus, B box, C terminal domain, which is a target for MAP kinase phosphorylation and the D domain located N terminus from the C box. Formation of the TCF-SRF-SRE requires protein-protein interaction mediated by the B box (Shore 1995) and protein-DNA interaction on the SRE site mediated by ETS domain (Dalton 1992). TCF are able to interact directly with Id2 via the ETS domain. HLH domain of Id2 is necessary for the interaction but N- and C-termini are necessary for the maximal effect (Yates 1999). Binding of Id2 to TCF disrupts ternary complex formation between TCF and SRF at the c-fos SRE *in vivo*. Association between Id2 and TCF factors can displace TCF factors from preformed protein-DNA ternary complex (Yates 1999). The mechanism of this inhibition suggests that allosteric effects could be involved instead of the simple blocking of the DNA binding surface. In the case of the c-fos promoter, where ERK-mediated induction of c-fos is mediated via ternary complex formation on SRE site, overexpression of Id2 causes a dose-dependent reduction of reporter activity in both c-fos promoter-linked and SRE-linked reporter constructs (Yates 1999). Because of the delayed expression pattern of Id2 compared to other immediate early gene, it could be involved in a negative feedback loop acting to downregulate genes such as c-fos or egr-1. Furthermore, TCF is activated by phosphorylation of its transcriptional activation domain by MAPK, although occupancy of the ETS motif within the c-fos SRE does not change following growth factor stimulation. Id2 protein could promote dissociation of phosphorylated and inactivated TCF. This would allow the replacement by unphosphorylated and inactivated form of TCF to reset the promotor.

One of the most interesting partners to Id2 is **SREBP-1/ADD-1, a bHLH-Lz factor**. Other members of the bHLH-Lz factors such as USF, c-Myc, AP4 and TFE3 enhancer of immunoglobulin heavy chain gene can not dimerise with Id2 (Sun 1991). ADD-1/SREBP-1 is unique in this respect. It co-immunoprecipitates with Id2 in *in vitro* in the absence of DNA (Moldes 1999). In adipocytes, ADD-1 binds strongly to E box site on the FAS promoter. Id2 translated in vitro can reduce this binding in a dose-dependent manner as shown by a gel shift experiment. In mature rat adipocytes, CAT reporter assays show that overexpression of Id2 dose dependently inhibits the activity of the FAS promoter (Moldes 1999). In adipocytes, USF and SREBP-1 mediate the activation of the FAS promoter by insulin (Wang 1997; Kim, Sarraf et al. 1998). They bind to the E box domain on FAS promotor in a mutually exclusive manner (Wang 1997). Differential interaction of SREBP-1 and USF with Id2 could affect the efficiency with which each factor is recruited to the FAS promoter. When a high level of Id2 is present, a smaller number of SREBP would be recruited to the promoter while the same number of USF would be recruited to the E box

sites. The changes in levels of transcriptional activators may affect the amplitude of gene expression. Other target genes of SREBP-1 such as glycerol-3-phosphate acyltransferase(Ericsson 1997), S14(Kim 1995), stearoyl CoA desaturase(Lopez 1996) or leptin (Kim, Sarraf et al. 1998) could be affected by the levels of Id2 proteins in a similar manner. This raises a possibility of a wider role of Id2 in the control of cellular lipids and cholesterol metabolism, which has not been investigated thoroughly to date.

Table 1.4 known binding partners of Id2

Binding partner	**Experimental system**	**Ref.**
Hypophosphorylated pRb P130, p107	*In vitro* translated pRb and GST tag Id2 *In vivo* co-transfected into Saos-2 cells	(Lasorella 1996)
Ubiquitous bHLH factors: E2A, E47 and E2B.m3		(Sun 1991)
Ubiquitous bHLH factors E2-2, E12	Yeast 2 hybrid assay	(Langlands 1997)
Ternary complex factor with ETS domain: Elk-1, SAP-1, SAP-2	*In vitro* GST pull down assay *In vivo* COS-7 cell co-transfection	(Yates 1999)
Neuronal tissue specific bHLH: Hes-1	*In vitro* mammalian two hybrid analysis *In vivo* co transfection in CHO cell	(Jogi 2002)
Myogenic HLH: MyoD, Myf-5. Mrf-4	Yeast 2 hybrid assay *In vitro* coimmunoprecipitation	(Langlands 1997)
SREBP-1/ADD-1 in adipocyte	*In vitro* co-immunoprecipitation	(Moldes 1999)

1.3.3.3 : Association of Id2 with retinoblastoma protein

In addition to TCF factors and SREBP-1, Id2 can form a heterodimer with the hypophosphorylated form of retinoblastoma protein. Id2 is the only member of the Id family with such capability as demonstrated by co-immunoprecipitation assays involving *in vitro* translated pRb and GST tag Ids (Iavarone 1994). Mutagenesis studies have shown that binding of Id2 to pRb occurs at the pocket domain of pRb (Iavarone 1994), a homologous domain that is required for the interaction of pRb with other cellular proteins including E2F, cyclin and MyoD. HLH domain of Id2 is essential for the binding (Iavarone 1994). The

same assay also shows that Id2 binds to pRb-related protein p107 and p130 *in vitro* and *in vivo* (Lasorella 1996). Once again the HLH region on Id2 is essential for the association. The functional significance of this association is discussed in more detail in section 1.3.3.7.

1.3.3.4 Regulation of Id2 expression

Id proteins are encoded by unlinked genes, as shown by chromosome mapping (Sun 1991). They exhibit unique expression pattern. Id1 and Id3 are widely expressed while expression of Id2 is restricted to specific tissues such as brain, liver, testis, muscle, haematopoietic cells and macrophages (Riechmann 1994). Analysis of the mouse Id2 promotor sequence in Figure 1.24 (EMBL/GenBank Data Libraries under the accession number AF077860 and AF077861) shows several motifs corresponding to known transcription factor binding sites. These include E box at bp 437, 582, 783 and 841. The Id2 promoter lacks an obvious TATA box (Mantani 1998). Sequences found at bp 791-1077 in the mouse Id2 gene correspond to a 300bp region in the human promoter. This region on the promotor is sufficient to repress the activity of a reporter gene in serum-starved cell and is required for the serum induction of Id2 expression. This region contains several known transcription factor binding sites. Sp1 consensus sequences are found at bp 343, 358, 704, 737 and 1018. The binding sites for ADR-1 and AP-4 as well as an E box are found at 841. These conserved cis-regulatory sequences are common to early-response genes whose expression is affected by mitogenic signals. In U87Y glioma cells this region appears to bind to repressor proteins present in serum starved cells but not in proliferating cells (Biggs 1995). By contrast in muscle C2 cells, the human Id2 promotor is regulated in response to both serum and protein kinase C activation through binding of ATF-like transcription factor as well as Sp1(Kurabayashi 1994).

The presence of many E boxes suggests the involvement of other bHLH factors in the regulation of Id2 expression. In fact, bHLH factors ME1 (HEB, REB, HTF-4), ME2 (E2A) and NSCL are able to promote Id2 promoter activity and Id2 itself is found to downregulate its own promoter activity (Neuman 1995). Additionally, Id2 expression is regulated at the transcriptional level by n-myc and c-myc and over expression of Id2 is accompanied by n-myc overexpression in many neuroblastoma cell lines (Lasorella 2000; Jogi 2002). More controversial are the ATF/CRE like sites located between -122 and -82. This region confers doxorubicin (Kurabayashi 1995) and forskolin -induced promoter activity(Scobey 2004) but neither ATF-1 nor CREB actually bind to this region in a band shift assay (Scobey 2004).

Because the second intron was not entirely sequenced, an additional DNA sequence from the 3'region of Id2 is placed in panel B. The putative polyadenylation signals are underlined.

Several polyadenylation signals (AATAAA) 3' to the coding region at 781, 877 and 910 bp are found, suggesting the presence of multiple RNA species encoding Id2 although northern blot analysis would not normally distinguish between these (Mantani 1998).

Generally in cell lines, the expression of Id proteins is highest during proliferation and lowest or absent in quiescent or terminally differentiated cells. Id protein levels are high in proliferating cells and tumour cells (Benezra 1990; Riechmann 1994)and is downregulated in myoblast and neuronal cells during terminal differentiation (Benezra 1990). The expression pattern of Id proteins, particularly Id2, in haematopoietic cells is an exception to the rule. Id2 is widely distributed in haematopoietic cells, with a high level of expression in the lymphoid cell line of both T and B cell origin as well as cells from the monocyte-macrophages lineage. The highest level is detected in the MC6 mastocytoma cell line (Cooper 1997). Levels of Id2 are low in all haematopoietic precursor cells except those of the monocyte-macrophage lineage. In contrast to other cell types, levels of Id2 are found to increase with progressing maturation of haematopoietic cells and is found to be up regulated during monocyte-macrophage differentiation (Ishiguro 1996; Cooper 1997). Unlike other cell types where changes in Id2 level correlate with changes in level of Id1 and cell proliferation, in haematopoietic cells, levels of Id2 correlate with cellular quiescence and shows inverse expression level in relation to Id1 (Cooper 1997). The reason for this differential regulation is unknown. However a few hypotheses may be suggested. Since (don't start with Because) haematopoietic cells undergo exit from the cell cycle at some point during their differentiation, an increase in Id2 levels may reflect a reduced proliferative capacity of these cells. Although an increase in level of Id2 might on the other hand reflect an attempt of these cells to continue cycling since Id2 has been shown to promote cell cycle through its interaction with pRb, Id2 may bind to other cell cycle-promoting factors, thereby preventing cell cycle progression.

The levels of Id proteins can be modulated acutely by environmental conditions in many cell types. For example, in 3T3 cells, NIH-3T3 cells and TIG-3 human diploid fibroblast, Id expression is induced by serum and PDGF (Christy 1991; Barone 1994; Hara 1994). Id2 is upregulated in C2 muscle cells in response to serum (Kurabayashi 1994) while level of Id1 is increased by serum in C2C12 and C3H10T1/2 fibroblast (Tournay 1996). An opposite observation is made in T-lymphocytes. Id2 is expressed constitutively in resting T-lymphocytes but downregulated in response to mitogenic stimulation (Ishiguro 1995). This regulation usually occurs at the mRNA level. The maximum response occurs around 1-3 hour post-stimulation, similar to the time course of other early response genes(Christy 1991; Biggs 1992; Deed 1993; Barone 1994; Hara 1994; Tournay 1996).

Figure 1.24 Id2 promoter

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A.

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   1 gaattcagaattaaagcattggagtcaagagctctaaacttttttcaaaatgtggctgcatctaggaagggtgctgaaaga
  81 ttccaaaacctcgtacgtaacagaatttttctttaaaaccagtgataagctgtcagtcaatagctaggaccacctacctga
 161 caaagagcttcccaagagctctaagtgttggaatgtgacaccagaaatcacgatttgtgcataattaatcgcatcacttt
 241 gccacctacactgaagggcacagaccaagggcagtgtatgtaaatgtagttccagtgtgcaaaccccactaatgaccttc
 321 gattaatggagtcattatagtaaccctgcctcattcttgggggtgggggagttccgaatgcaccgggtccctcggggct
 401 cctctgcggtctgagggagaccgcacagtgttcctacaattgtgtcactgagtttccgagaaggcctcccgcgttgctcc
 481 aagttgcaaagcttcacgctaaacctgtcatggacgtgtatgtgggtaggttccccccctcccaaatagctttccttt
 561 atcttaaaaggaggaacactacaaatgtgacttcccaaaagcgcgcttctttttcccttctgccacggtcgctgtcttccc
 641 taacgctagcgacctgtgacttcccgtctgctgtgcggagacagtggggctgctggaagtcctgggggcgggtatagcta
 721 gctccgggcacacccccgggcagggtcctgccagctctgggaattggaataaggcgggcacaattgcacactcaggcc
 801 ccctgggcgcccagcgcacactgtactcaatttgccaccccagctgggctctcgcccgctcctcccaccctacaggtatt
 881 ggctgcgaacgcggaagaaccgagagctcatactcaccaatgggagaattcgcctggtatgatggacgggagcccttcca
 961 ccaatggcaattcagggatgcccgattgagcggccagggcgagtgcacataaaagacgccccgcccggctcgcgcttcat
                                                                                    M
1041 tctGAACCGAGCCTGGTGCCGCGCAGTCAGCTCAGCCCCCTGTGGCGGCTCCCTCCCGGTCTCTCCTCCTACGAGCAGCA
       K  A  F  S  P  V  R  S  V  R  K  N  S  L  S  D  H  S  L  G  I  S  R  S  K  T
1121 TGAAAGCCTTCAGTCCGGTGAGGTCCGTTAGGAAAAACAGCCTGTCGGACCACAGCTTGGGCATCTCCCGGAGCAAAACC
     P  V  D  D  P  M  S  L  L  Y  N  M  N  D  C  Y  S  K  L  K  E  L  V  P  S  I  P
1201 CCGGTGGACGACCCGATGAGTCTGCTCTACAACATGAACGACTGCTACTCCAAGCTCAAGGAACTGGTGCCCAGCATCCC
       Q  N  K  K  V  T  K  M  E  I  L  Q  H  V  I  D  Y  I  L  D  L  Q  I  A  L  D  S
1281 CCAGAACAAGAAGGTGACCAAGATGGAAATCCTGCAGCACGTCATCGATTACATCTTGGACCTGCAGATCGCCCTGGACT
       H  P  T  I  V  S  L  H  H  Q  R  P  G  Q  N  Q  A  S  R  T  P  L  T  T  L  N
1361 CGCATCCCACTATCGTCAGCCTGCATCACCAGAGACCTGGACAGAACCAGGCGTCCAGGACGCCGCTGACCACCCTGAAC
     T  D  I  S  I  L  S  L  Q
1441 ACGGACATCAGCATCCTGTCCTTGCAGgtgagactagcttgcaagtacgccactgcccagacgctccgggtctcccgagc
1521 tgtcactcttaaagcccatcgtagagacaggttcattaactttatttttgaggaaactgtatattgagcgtcatgtgaaa
1601 tcgctacttataagttctgtgtgggttgcgtctggatctgcgctgtagcatgatcctgtttcatgggacttgttggcact
                                                                                  X  S
1681 tttgtgaaaggaggaggggggctaccttcctttaagattacctaatatcctgccttttatcctctttctccccagGCAT
       E  E  P  S  E  L  M  S  N  D  S  K  V  L  C  G  *
1761 CTGAATTCCCTTCTGAGCTTATGTCGAATGATAGCAAAGTACTCTGTGGCTAAATAAATggtgagtgttgcgggtgcctc
1841 ctgtgtgcgcgtttcggtaatgtgcttgtgtgtctgttaaatgtttggtttggtaaatgcatgcttacttcactgtgtta
1921 cgggtgccgcttgacttaccacataggcatgaaggggcttgtagctgtggttgctcaggactacagaacagttgccttta
2001 caaaaacagaaagaaagaaagaaagaaagaaagaaagaaatgccagtaacttactatgaaggtgtcaggaccaagtgtggct
1081 gacttttatgagcccagtggcggtacaatgaggtgctaacctcagctgtaattaatcttatcgccacaaattccatagtg
2161 attctcttttcccaaaactgtagccctctgagatttctactggctagctgaagacggataccccgagccttacccatgtg
2241 ctctgggatcc
```

B.

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   1 gtggttcttcggcgccaggttcgcccgcttctgcccttaggtaacattctctaaactgcgtttctctcccaatcttttgc
  81 agGCATTTGGGGACTTTTTCTTTTCTTTTTACTTTCTCTTTTTCTTTTGCACAAGAAGAAGTCTACAAGATCTTTTAAGA
 161 CTTTTGTTATCAGCCATTTCACCAGGAGAACACGTTGAATGGACCTTTTTAAAAAGAAAGCGGAAGGAAAACTAAGGATG
 321 ATCGTCTTGCCCAGGTGTCTTGTTCTCCGGCCTGGACTGTGATACCGTTATTTATGAGAGACTTTCAGTGCCCTTTCTAC
 401 AGTTGGAAGGTTTTCTTTATATACTATTCCCACCATGGGGAGCGAAAAGGTTAAAAAAAAGAAAAAAATCACAAGGAATT
 481 GCCCAATGTAAGCAGACTTTGCCTTTTCACAAAGGTGGAGCGTGAATTCCAGGAGGACCCAGTATTCGGTTACTTAAATG
 561 AAGTCTTCGGTCAGAAATGGCCTTTTTGACACGAGCCTACTGAATGCTGTGTATATATTTATATATAAATATATATATAT
 641 TGAGTGAACCTTGTGGACTCTTTAATTAGAGTTTTCTTGTATAGTGGCAGAAATAACCTATTTCTGCATTAAAATGTAAT
 721 GACGTACTTATGCTAAACTTTTTATAAAAGTTTAGTTGTAAACTTAACCCTTTTATACAAAATAAATCAAGTGTGTTTAT
 801 TGAATGTTGATTGCTTGCTTTATTTCAGACAACCAGTGCTTTGATTTTTTTTTATGCTATGTTATAACTGAACCCAAATA
 881 AATACCAGTTCAAATTTATGTAGACTGTATTAAGATTATAATAAAATGTGTCTGACATCAAtgccgggatctgaatggtt
 961 tttgaagtgtcttgtgtttctggggcactcc
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In addition to serum, Id2 can be induced in response to insulin-like growth factor (IGF) (Barone 1994; Baudino 2001; Belletti 2001; Navarro 2001; Prisco 2001). The response of Id2 to IGF-1 has been studied extensively in 32D murine haematopoietic cell line over expressing IGF-1R where Id2 mediated the proliferative response to IGF. Wild-type 32D cells require IL-3 for survival. Without IL-3, 32D cells undergo apoptosis (Askew 1991). With the expression of IGF-IR, these cells become IL-3-independent for survival and can be stimulated to grow exponentially for 48 hour before they eventually differentiate along the granulocyte pathway (Peruzzi 1999; Valentinis 1999; Dews 2000; Valentinis 2000). Exponentially growing 32D-IGF-1-R cells stimulated with IGF show an increase in Id2 mRNA and protein level (Belletti 2001). The increase in Id2 expression requires both PI3K

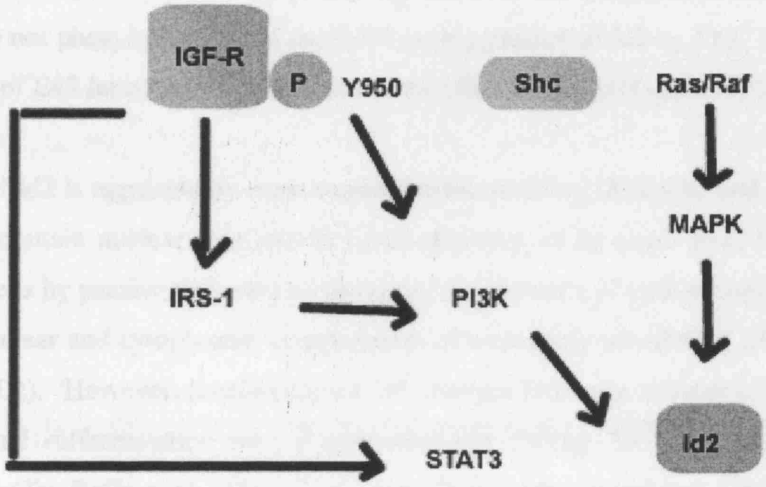
and MAPK. The requirement for PI3 kinase has been demonstrated by a reduction in Id2 expression in LY294002 treated cells and the enhancement of IGF-1-mediated induction in 32D-IGF/IRS-1 cells expressing wild type IRS-1(Belletti 2001). The requirement for MAPK has been demonstrated by a reduction in Id2 expression in PD98059 treated cells as well as a reduction in response in cells carrying Y950 mutation in the IGF-1 receptor (Prisco 2001). These observations suggest that Y950 in 32D cell sends dual signals through both MAPK and PI3K pathways. Binding of Y950 to Shc protein (Craparo 1995; Tartare-Deckert 1996) sends signals through Ras/Raf/MAPK pathway (Basu 1994) while activation of IRS-1 sends signals through the PI3 kinase pathway. The end result of the upregulation of Id2 protein in this system is the inhibition of differentiation of 32D cells and in the case of dominant negative STAT3 expressing cell, an increase in growth rate and malignant transformation.

In haematopoietic cells, differentiating growth factors send two simultaneous signals, one for proliferation and another for differentiation. Likewise, the process of differentiation in 32D-IGF-1-R cells is preceded by a short intense period of proliferation mediated by Id2 before the cells eventually differentiate along the granulocyte pathway (Valtieri 1987). In 32D-IGF-R cells, the overexpression of dominant negative STAT3 abolishes IGF-mediated differentiation and causes a significant increase in levels of Id2 (Belletti 2001). The observation suggests that in these cells, Y950 simultaneously induces differentiating signals through STAT3 by phosphorylating STAT3 on Y705. Eventually the activation of STAT3 phosphorylation inhibits Id2 expression through an unknown mechanism. This leads to terminal differentiation of 32D cells.

Another regulator of Id2 expression is TGF-β. It inhibits Id2 expression in HaCaT keratinocyte and NMuMG and mouse mammary epithelial cells. The reduction coincides with an increase in Mad2 and Mad4, the antagonistic repressors of Myc. The Mad-Max inhibitor complex replaces Myc-Max activator complex on the E-box of the Id2 promoter in response to TGF-β (Siegel 2003). Interestingly, the effects of TGF-β are tissue-specific. TGF-β actually induces Id2 gene expression in dendritic cells and in early as well as mature B cells (Sugai 2003). In B cell, the induction of Id2 by TGF-β results in an inhibition of IgE class switch recombinant (Sugai 2003). Id2 affects this process at the level of the germline transcription. When Id2 is removed, for example in B cells of Id2 knockout mice, activity of E2A and Pax5 increases. They bind to S_E germline promoter and augment the expression of $_E$germline transcription. Since the efficiency of class switch recombination is correlated with the quantity of germline transcription (Lee, Kinoshita et al. 2001), Id2-deficient B cells undergo class switching recombination to IgE at a much more frequent rate than wild type B cells. An excessive amount of IgE leads to allergic response such as asthma, anaphylaxis and

hay fever although IgE protects against some parasites. Therefore, its level is kept comparatively low compared with other Ig isotypes (Gould, Sutton et al. 2003). By inhibiting the S_E germline promoter, Id2 contributes directly to the inhibition of the allergic response.

Figure 1.25 control of Id2 expression by IGF-1



Even in terminally differentiated cells, Id2 expression is responsive to hormonal stimuli and metabolic second messengers. For example, in terminally differentiated sertoli cells, Id2 expression is induced by serum. Overexpression of Id2 protein in these cells leads to an increase in transferrin promoter activity. This observation points to the role of Id2 in the maintenance of differentiated phenotype in Sertoli cells in response to FSH and in maintaining growth potential in response to serum (Chaudhary 2001). Additionally, Id2 expression in sertoli cells is induced by forskolin or follicle-stimulating hormone as well as the overexpression of protein kinase A. The Id2 mRNA level starts to increase after 2 hour, reaching its maximum level after 6 hour and decreases down to basal after 24 hours(Scobey 2004). The decrease might be the effect of a negative feedback, with Id2 binding to another E box binding protein necessary for its expression, resulting in the formation of an inactive heterodimer.

In adipocytes, Id2 is downregulated with differentiation. However, Id2 mRNA level can be re-induced by cAMP raising agents such as isoproterenol and IBMX (Moldes 1999). The level of cAMP could control the balance of Id2-SREBP-1c heterodimer in this system and consequently Id2 will provide an alternative control mechanism for the SREBP-1c transcription mechanism. Although Moldes et al. have shown association of Id2 and SREBP-1c *in vitro* and showed that overexpression of Id2 led to a reduction in FAS gene activity in 3T3-L1 cell, they did not demonstrate that the increase in Id2 as a result of β-adrenergic

pathway activation actually alters heterodimer ratio or affects promoter activity in adipocytes. It remains unclear whether a similar mechanism operates in other cell types or other SREBP dependent promoters.

1.3.3.5 Post translational regulation of Id2

Id2 can be phosphorylated *in vitro* by cAMP-dependent protein kinase, cdc2 kinase and protein kinase C. However, neither phosphorylation of Id2 protein by cAMP dependent protein kinase nor phosphorylation of the E47 binding partner of Id2 by PKC actually affects the inhibition of E47 homodimer formation and its DNA binding (Nagata 1995).

The activity of Id2 is regulated by nucleo-cytoplasmic shuttling (Kurooka and Yokota 2005). Id2 does not contain nuclear localisation signal. Because of its small size, Id proteins can enter the nucleus by passive diffusion as shown by the presence of epitope tagged Id2 protein in both the nuclear and cytoplasmic compartment of transiently transfected COS cells (Ghil, Jeon et al. 2002). However, localisation of Id2 changes from the nucleus to the cytoplasm during neuronal differentiation into oligodendrocytes (Wang 2001) and during myeloid differentiation (Tu, Baffa et al. 2003). This suggests that other regulatory mechanisms exist. In fact, Id2 contains the leucine-rich consensus nuclear exclusion signal on the C terminus. This signal directs Id2 for nuclear export by the nuclear export receptor chromosome region maintenance-1 (CRM1). It directs cytoplasmic localisation of Id2 under the conditions in which passive diffusion is inhibited. Whilst the C terminus of Id2 is important for nuclear exclusion, mutagenesis studies reveal that nuclear localisation of Id2 requires the HLH domain. Mutation in this domain severely compromises nuclear import although Id2 retains the ability to bind to E proteins. The association with other proteins such as p204 through its HLH domain in muscle cells may sequester Id2 from the nucleus by masking nuclear import signal on this HLH domain (Liu, Ding et al. 2002).

Id2 is degraded through the ubiquitin-proteasome degradation pathway (Bounpheng, Dimas et al. 1999). It has a very short half life of around 15 minutes. It is especially targeted through N-terminal ubiquitination instead of ubiquitination on the NH2 group of an internal Lys residue (Fajerman, Schwartz et al. 2004). The formation of ubiquitin conjugates requires the sequential action of three enzymes; E1, E2 ubiquitin conjugate and E3 ubiquitin ligase. The successive ligation lengthens the polyubiquitin chain that serves as a degradation signal for the proteasome. The process of N-terminal ubiquitination does not require an internal Lys residue. Instead, N-terminal ubiquitination occurs on the N-terminal residue. This is followed by recognition of downstream motifs by the E3 subunit. Subsequently, this is followed by the anchoring of canonical Lys_{48} polymerised ubiquitin chain to the Lys_{48} of the

N-terminally conjugated ubiquitin (Johnson, Ma et al. 1995). The use of nonhydrolysable analogues of ATP demonstrates that the conjugation of ubiquitin to Id2 requires ATP but the phosphorylation of residues on Id2 is not necessary for its ubiquitination in a cell free system (Fajerman, Schwartz et al. 2004). Nonetheless, in an *in vivo* model, Id2 levels decrease after it is phosphorylated on Ser 5 by CDK2 (Hara 1997).

1.3.3.6 Id2 and differentiation

In various cell types differentiation is controlled by a combination of bHLH transcription factors as revealed by gain of function and loss of function experiments (Olsen 1994; Kageyama 1997; Massari 2000). Tissue specific bHLH factors such as MyoD generally form dimers with ubiquitously expressed E proteins consisting of products of E2A, HEB and E2-2 genes(Massari 2000). Id proteins are able to inhibit differentiation by forming inactive heterodimers with these E proteins as has happened in models of myogenesis and myelopoiesis (Norton 1998). During skeletal myogenesis, the levels of Id2 decrease, allowing myogenin, a bHLH factor, to form transcriptionally active heterodimers on caveolin-3 promotor. Proximal E box elements serve as myogenin binding sites and is both necessary and sufficient to control caveolin-3 gene transcription as is the case in C2C12 cells (Biederer 2000).

Id2 also lays at the heart of the mechanism which links proliferation to differentiation in developing oligodendrocytes. Id2 overexpression inhibits oligodendrocyte development from OPCs and endogenous Id2 translocates out of the nucleus into the cytoplasm prior to the onset of differentiation. In the presence of mitogen, OPC with Id2(-/-) proliferates much more slowly and differentiates prematurely compared to wild-type cells in response to T3 stimulation (Wang 2001). Bone morphogenetic protein (BMP) is another factor that inhibits the generation of oligodendroglia and enhances generation of astrocytes by neural progenitor cells. BMP increases the expression level of Id2 and Id4, leading to sequestration of basic helix loop helix loop factor OLIG, which is required for the generation of oligodendrocytes (Samanta and Kessler 2004). Generally Id2 expression decreases during neurogenesis and myogenesis.

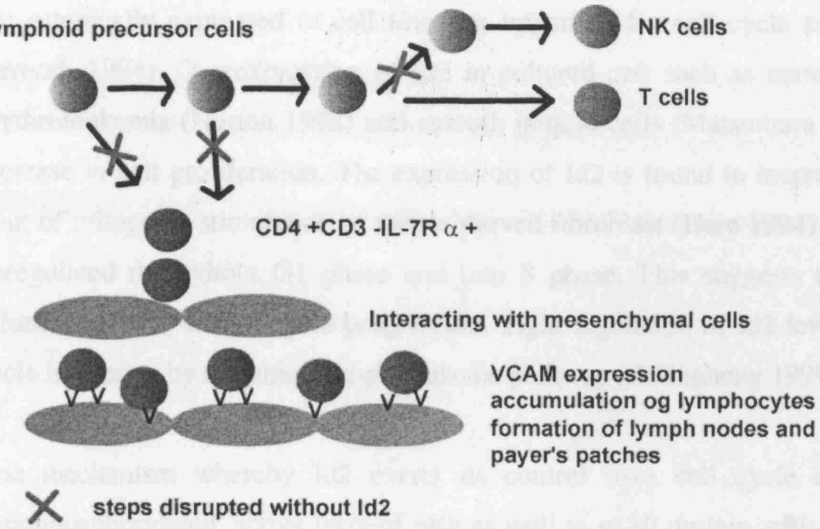
The bHLH factors are also involved in haematopoietic differentiation. E2A forms heterodimer with myeloid and erythroid specific tal-1 *in vivo*. This heterodimer represses promoters containing CAnnTG E box sequence. When M1 myeloid cells are induced to differentiate terminally into macrophages with the cytokine IL-6, the binding of E2A-tal-1 heterodimer to the CAnnTG DNA motif decreases. This event coincides with an increase in Id2 expression and recombinant Id2 is able to reduce E2A-tal-1 complex DNA binding in an *in vitro* system (Voronova 1994). In conclusion, during myeloid cell differentiation, Id2

inhibits binding of E2A-tal-1 repressor complex, thereby allowing the expression of an unknown protein vital for myeloid differentiation. In macrophages, Id2 level is sustained throughout differentiation. In this respect Id2 expression during monocyte/macrophages differentiation differs from the regulation in neurogenesis and myogenesis.

Id2 expression also affects the other aspects of the immune system. In Id2-deficient mice (Id2 -/-), peripheral lymphoid organs fail to develop normally. They lack the lymph nodes and Peyer's patches. Flow cytometry and *in situ* hybridization fails to identify lymphotoxin system receptor expressing cells of the specific population of CD45+CD4+CD3-IL-7Rα+ necessary for the development of peripheral lymphoid organs (Fu 1999; Yokota 1999). Id2(-/-) mice also lack natural killer cells that participate in innate immunity and in tumour rejection in spleen, thymus, liver and bone marrow. Together the animal demonstrates a systemic defect in NK cell development (Yokota 1999; Ikawa 2001). The bone marrow of these mice fails to develop into NK cells in the presence of IL-15 *in vitro* (Yokota 1999). Further studies show that Id2 plays a vital role in cell fate determination towards NK lineage cells at the point where NK and T lineage cells bifurcate from a common precursor (Ikawa 2001). Biochemical study shows that E2A gene product and HEB, both able to form heterodimer with Id2, are crucial for T cell development (Morrow 1999). Id2 seems to restrict cell fate of bipotent T/NK precursor to NK cells by inactivating activity of these E proteins. The same occurs in B cells. Id2 is downregulated during differentiation of immature B cell into mature B2 and MZ B cells. High levels of Id2 inhibit binding of E2A to the E2 box site (Becker-Herman 2002).

Other than the immune system, Id2 is involved in the development of mammary gland. During pregnancy, mammary glands of Id2 (-/-) mice fail to expand and mammary epithelial cells display proliferation arrest even when grafted into wild-type mammary gland. This observation rules out inadequate hormonal environment or mesenchymal factors as causes (Mori 2000). These cells, however, never display signs of premature differentiation and remain in the immature state and elevation in the level of cyclin dependent kinase inhibitor $p27^{kip1}$ and $p21^{WAF1}$ but not $p16^{INK4a}$ is detected (Mori 2000). Since the over expression of E2A in cultured cells leads to the enhancement of $p21^{WAF1}$ cell cycle arrest, which is blocked by Id1(Peverali 1994; Prabhu 1997), a similar mechanism could underline the enhancement of $p27^{kip1}$. Loss of Id2 could lead to functional upregulation of a bHLH factor, leading to the upregulation of cyclin-dependent kinase inhibitors although a mammary gland-specific bHLH factor has not been identified.

Figure 1.26 Id2 and immune cell differentiation



Id2 has recently been associated with the self-renewal process of mouse embryonic stem cells (ES cells). It is generally accepted that LIF and other gp130 receptor acting cytokines are essential for the maintenance of self-renewal characteristics of ES cells (Smith 2001), which are characterised by undifferentiated phenotype, pluripotency and embryo colonisation capacity. Self-renewal requires signals from LIF and an additional signal from bone morphogenetic proteins (BMPs) (Ying 2003). LIF mainly acts through STAT3 activation (Matsuda 1999)while the principal downstream effectors of BMPs receptors are Smad transcription factors R-Smad and Smad 1, 5, 8 (Attisano 2002). Smads are known to increase Id protein expression in C2C12 myoblasts (Nakashima 2001) and neuroepithelial cells (Lopez-Rovira 2002). In ES cells Id1, Id2 and Id3 expression are increased by BMPs as well as LIF in combination with BMPs. Id overexpression can substitute for the effect of BMP in combination with LIF(Ying 2003) but Id overexpression alone is not able to maintain self-renewal without LIF. Without LIF, ES cells differentiate into non-neuronal cells. An experiment involving a revertible Id expression construct and an overexpression of E47 (Ying 2003) shows that Id proteins are involved with the blockade of lineage specific bHLH transcription factors such as mash1, thereby limiting the formation of proneuronal heterodimers. Precisely how Id proteins exert lineage restriction in ES cell differentiation remains unanswered.

1.3.3.7 Id2 and cell cycle control

In many cell types, a certain level of Id proteins is required for propagation (Norton 2000). Ablation of Id protein expression and function by antisense oligonucleotide blockade (Barone 1994; Hara 1994) or by antibody-microinjection strategies (Peverali 1994) has

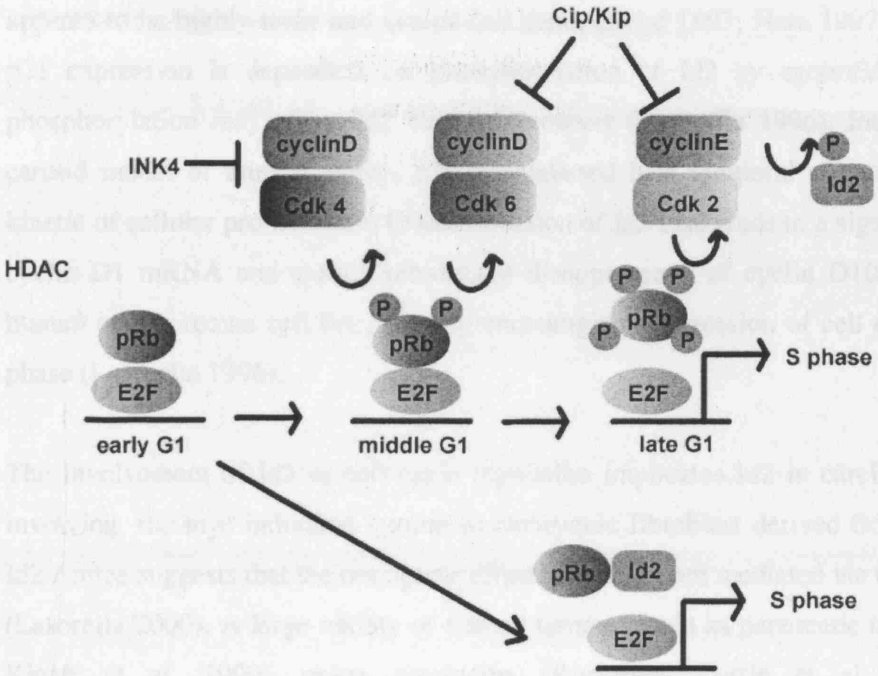
implicated the requirement for Ids function in the G1-to S phase progression. This observation is in accord with the fact that other HLH factors such as E47 or myoD, which are ectopically expressed in cell line, are important for cell cycle arrest(Sorrentino 1990; Peverali 1994). Overexpression of Id2 in cultured cell such as mammary epithelial cells, erythroleukemia (Norton 1998) and smooth muscle cells (Matsumura 2002) also lead to an increase in cell proliferation. The expression of Id2 is found to increase rapidly within 1-2 hour of mitogenic stimulation of serum-starved fibroblast (Hara 1994). Id2 levels are further upregulated throughout G1 phase and into S phase. This suggests that Id2 could play a bifunctional role in cell cycle progression. Tight regulation of Id2 level throughout the cell cycle is exerted by the ubiquitin-proteasome pathway (Bounpheng 1999).

One mechanism whereby Id2 exerts its control over cell cycle is by binding to the hypophosphorylated, active form of pRb as well as p130 protein. pRb suppresses growth by interacting with growth promoting intracellular proteins such as E2F and D-type cyclin (Harbour 2000). The activity of pRb is regulated through phosphorylation by a series of cyclin-dependent kinases (CDK) including CDK4 and CDK6 acting with cyclin D1, D2 and D3 as well as CDK2 acting with cyclin E and cyclin A. The current view is that CDK4 phosphorylates the C terminus of pRb, leading to successive intra molecular interactions that eventually displace the pocket domain from histone deacetylase. Consequently, pRb loses its ability to repress transcription. This then leads to the phosphorylation of pRb by CDK2. This phosphorylation inhibits pRb from binding to and inhibiting E2F transcription factor whose activity is required for an entry into S phase.

Following growth factor stimulation of arrested cell, Id2 level shows a biphasic pattern in early and late G1 phase of the cell cycle (Barone 1994; Hara 1994). A possible explanation for this observation is the sequential inactivation of distinct targets throughout the progression of cell cycle during the G1 phase. The first peak of Id2 expression following mitogenic signal stimulation may disrupt the p130-E2F4/5 complexes which are believed to be the inhibitory complex for cell growth in S phase(Hansen 2001). The second peak of Id2 expression in G1 occurs when E2F1-3 is actively repressed by pRb and p107 (Humbert 2000). Disruption of this complex by the binding of Id2 to pRb and p107 would release the E2F1-3 from pRb/p107. This leads to the expression of genes required for G1-S phase progression. In support of this model, pRb-mediated cell cycle arrest in Saos-2 cell is reversed by Id2. The same is observed with p107 and p130 mediated arrest in Saos-2 cells (Lasorella 1996). Rb(-/-) mice are embryonic lethal, displaying widespread cell proliferation, defective differentiation and apoptosis in the central nervous system and the haematopoietic system. However, a double Rb(-/-)Id2(-/-) mice survive and do not display the same

anomalies. The functional rescue of Rb deficiency in the absence of Id2 implicates the correct balance of Id2 and pRb in the control of cell proliferation (Lasorella 2000).

Figure 1.27 Id2 control of G1-s phase progression



Id2 also affects other cell cycle regulatory proteins in addition to pRb. As a critical regulator of mitogenic signalling pathways, activities of CDK are regulated at multiple levels, including the synthesis and the stability of each component, the assembly and the post translational modification and the control of complex formation with cyclin-dependent inhibitors (CKIs). The component affected by Id2 is CKI. CKI inhibits cyclin-cdk activity and enhances pRb activity. It acts as a growth inhibitor in many cell types. There are two classes of CKI, the Kip/Cip family (p21, p15 and p57) and the INK4 family (p16, p15, p18, p19). The INK4 family specifically binds to and inactivates CDK4 and CDK6. The over expression of Id2 reverses p16 and p21 mediated cell cycle arrest in human osteosarcoma cell line (Lasorella 1996). The inhibition occurs through transcriptional repression of the p21 gene. E2A has the ability to block cell cycle progression partly through the transcriptional activation of p21 gene. Id1 binds to E2A and inhibits this activity (Prabhu 1997). This causes an increase in cyclin-cdk activity, which leads to a reduction in the number of activated pRb. This mechanism is important in the enhancement of smooth muscle growth in response to Id2.

Post-translational modification of Id2 also contributes to cell cycle control. Id2 contains phosphorylation sites for cyclin E and cyclin A/Cdk-2 at Ser5. During cell cycle progression,

Id2 becomes phosphorylated at these sites; the phosphorylation process coincides with the appearance of cyclin A and E-type cyclin during G1/early S phase. The phosphorylated form of Id2 exists until the end of S phase (Hara 1997). Although the exact function of these phosphorylated forms is not known, they are vital since the non-phosphorylatable mutants appears to be highly toxic and causes cell death (Deed 1997; Hara 1997). The inhibition of p21 expression is dependent on phosphorylation of Id2 by cyclinE/cdk2. In this case phosphorylation may affect Id2 nuclear transport (Lasorella 1996). Interestingly, in a rat carotid model of arterial injury, Id2 is expressed in a temporal pattern that parallels the kinetic of cellular proliferation. Overexpression of Id2 also leads to a significant reduction in cyclin D1 mRNA and causes subsequent disappearance of cyclin D10cdk4 complexes in human osteosarcoma cell line, thereby encouraging progression of cell cycle from G1 to S phase (Lasorella 1996).

The involvement of Id2 in cell cycle regulation implicates Id2 in carcinogenesis. A study involving the myc induction system in embryonic fibroblast derived from Id2-/- and Rb-/- Id2-/-mice suggests that the oncogenic effects of c-Myc are mediated via the Id2-Rb pathway (Lasorella 2000). A large variety of human tumours such as pancreatic tumour (Maruyama, Kleeff et al. 1999), colon carcinoma (Rockman, Currie et al. 2001), colorectal adenocarcinoma (Wilson, Deed et al. 2001), astrocytic tumour (Vandeputte, Troost et al. 2002), Ewing sarcoma (Fukuma, Okita et al. 2003) and squamous cells carcinoma (Langlands, Down et al. 2000) display increased expression of Id2 proteins. Id2 expression has been correlated with high malignancy in astrocytes (Ruiz, Huang et al. 2004) and it is involved in the initiation, growth and angiogenesis of pituitary tumour by functioning as stimulator of VEGF expression (Lasorella, Rothschild et al. 2005).

1.3.3.8 Id2 and atherosclerosis

Causative links between Id2 and atherogenesis have been suggested by findings in animal models. In Id2 and ApoE double knockout mice, there is a 40% reduction in atheromatous area in aorta compared with ApoE single knockout mice after 12 weeks of high fat diet (Aoki 2003). The finding suggests that Id2 could contribute to lesion development. Upregulation of Id2 has been found in many arterial cells involved with atherosclerosis development. A microarray study shows higher level of Id2 mRNA in hypoxia-affected macrophages (Burke 2003). Hypoxia promotes accumulation of these macrophages in ischaemic areas of atherosclerotic plaques through an unknown mechanism and the effects of Id2 upregulation on cellular metabolism in this context are not known. Id2 upregulation in these cells could be mediated through hypoxia-inducible factor-1 as in hypoxic neuroblastoma cells and human breast cancer cells MCF-7 (Lofstedt, Jogi et al. 2004).

Upregulation of Id2 has been found in smooth muscle cells in the rat model of carotid injury (Matsumura 2002). This increase may facilitate pathological proliferation of SMC in the lesion. Finally, in the ob/ob mouse model of obesity-linked type II diabetes, Id2 mRNA is up regulated in skeletal muscle, liver and fat. This increase in expression might play a profound role in the pathogenesis of type II diabetes through a yet unresolved intracellular mechanism (Vicent 1998).

Aim

1) To explain the link between hyperglycaemia, hyperinsulinaemia and hyperleptinaemia with changes in HSL levels

2) To find new glucose responsive gene in J774.2 macrophages

Chapter 2 Materials and Methods

2.1 Materials

J774.2 cells, L6 cells, HepG2 cells, IgG 2A4 hybridoma cells were obtained from the European Tissue Culture Collection, and 3T3-L1 fibroblasts were obtained from the American Tissue Culture Collection. Rat cardiomyocytes were kindly provided by Dr. Hillary and rat soleus muscles were kindly provided by Dr. Jorgen Jensen , Oslo. RPMI 1640 media with and without glucose, antibiotic antimycotic solution, glutamine, newborn calf serum were obtained from Gibco. DMEM media with 5mM and 20mM glucose and fetal calf serum were obtained from Sigma. Anti Id2 antibody was obtained from Santa Cruz Biotechnology. Anti phospho-Erk antibody, anti total Erk antibody, anti phospho-CREB antibody, anti total CREB antibody, anti c-Jun, anti phospho -PKB (ser 473) antibody, anti total PKB antibody and anti-pan PKC antibody were obtained from Cell Signalling Technology. RL-2 antibody was obtained from PIERCE. Dual luciferase assay kit was obtained from Promega. O-link glycosylation detection kit was obtained from PIERCE. Fugene 6 transfection kit was obtained from Roche Applied Sciences. Lipofectamine transfection kit was obtained from Invitrogen. Protein-G agarose, 2-deoxyglucose, 3-o-methyl-glucose, glucosamine, 8-cpt-cAMP, azaserine, adrenaline, IBMX, insulin, lysophosphatidic acid, TBS, Tween-20, aprotinin, pepstatin, leupeptin were obtained from Sigma Aldrich. The Epac specific cAMP analogue was obtained from Biolog. Murine Leptin was obtained from R&D Systems. Wortmannin, PD98059, LY294002, H89, AEBSF, ALLN were obtained from Calbiochem. SB 415286 and SB 216763 were obtained from Tocris. D-glucose was obtained from BDH. Id2 expression plasmids were kindly provided by Dr. Hara (University of Tokyo). pCMV SREBP-1 plasmids and pGL3 basic (HSL promoter -2682/+301) were kindly provided by Dr. K.T. Dalen (University of Oslo). pGL3 enhancer (HSL promoter -86/+45) plasmids were kindly provided by Dr. Palmer (University College London, UK). Empty pGL3 basic, pGL3 enhancer, pGL3 enhancer promoter were kindly provided by Dr. E. Shephard, UCL. Empty pcDNA 3 plasmids were kindly provided by Dr L. Foukas. Bicinchoninic Acid (BCA) Protein Assay kit was obtained from PIERCE Chemical company (Pierce-Warriner).

2.2 Methods

2.2.1 Cell culture

All reagents were pre-warmed to 37° C and all procedures were carried out in a class II laminar flow hood, providing a sterile environment. All cells were maintained at 37° C in 5% CO_2 gassed incubator.

2.2.1.1 J774.2 cells (Ralph, 1975) are a macrophage -like cell line with the macrophage properties of adherence, morphology, receptors for immunoglobulin and antibody dependant lysis of target cells (Ralph, 1976). They are semi-adherent and both floating and attached populations show identical property (Ralph, 1975). J774 are commonly used as a foam cell model (Miura, 1997;Bernard, 1991;Avart, 1999) and a model for macrophage cholesterol ester metabolism studies (O' Rourke, 2001;O'Rourke, 2002). J774.2 macrophages were grown in RPMI 1640 media (10mM glucose) supplemented with 10% fetal calf serum (heat inactivated at 50°C for 30 minutes) and 1% antibiotic-antimycotic. The medium was removed and replaced with fresh media every 48 hours. To passage the cell medium was removed and cell mono-layer was scraped with a plastic scraper before being resuspended in an appropriate amount of pre-warmed media. Suspended cells were divided into an appropriate number of dishes.

2.2.1.2 3T3-L1 fibroblasts can be differentiated into 3T3-L1 adipocytes after they become confluent. During differentiation the cell develop adipocyte-like morphology by becoming rounded and forming lipid droplets (Green, 1974). 3T3-L1 adipocytes are very responsive to insulin and show increased glucose transport and glycogen synthesis and reduced lypolysis upon stimulation with insulin. 3T3-L1 fibroblasts were grown in DMEM media with 4.5g/l glucose supplemented with 10% NBCS and 1% antibiotic antimycotic. To passage the cells medium was removed, monolayer was washed with 5 ml sterile PBS containing Mg^{++} and Ca^{++} before an addition of 1ml of trypsin/EDTA per 75cm^2 flask. Cells were incubated at 37° C for 5-10 minutes then the flask was tapped to release cells from the surface. An appropriate amount of medium was added and suspended cells were divided into corresponding number of dishes. The differentiation method followed was that of Frost and Lane{Frost, 1985 #10}. Briefly two days after cells were confluent, old medium was removed and fresh DMEM media (4.5g/l glucose) supplemented with 10% FCS, 1% antibiotic-antimycotic, 1μg/ml insulin, 0.25μM Dexamethazone and 0.5mM IBMX (3-isobutyl-1-methyl-xanthine) was added to the cells. On day 4 old medium was removed and

fresh DMEM media (4.5g/l glucose) supplemented with 10% FCS, 1% antibiotic-antimycotic, and 1μg/ml insulin was added to the cells. On day 6 old medium was removed and fresh DMEM media (4.5g/l glucose) supplemented with 10% FCS, and 1% antibiotic-antimycotic was added to the cells and the cells were fed with this media every 48 hours thereafter. 3T3-L1 adipocytes were used for experiment between day 7 and day 10.

2.2.1.3 L6 myoblasts are a rat thigh muscle cell line. Cells grow as myocytes but can differentiate into myotubes if confluency is maintained for 7 days. The **Hep G2 cell line** was been isolated from a human liver with a well differentiated hepatocellular carcinoma. It has been grown successfully in large scale cultivation systems and responds well to hormonal treatment. L6 myoblasts and HepG2 cells were grown in DMEM media with 4.5g/l glucose supplemented with 10% FCS and 1% antibiotic antimycotic. To passage the cells medium was removed, the monolayer was washed with 5 ml sterile PBS without magnesium and calcium before an addition of 1ml of trypsin/EDTA per 75cm^2 flask . Cells were incubated at 37°C for 5-10 minutes then the flask was tapped to release cells from the surface. An appropriate amount of medium was added and suspended cells were divided into an appropriate number of dishes.

2.2.1.4 Cryo-preservation of cells

Cells were removed from the surface of tissue culture flasks by scraping or trypsinisation then resuspended in 5ml of suitable media without serum or antibiotic. Cells were pelleted by centrifugation at 1200rpm for 10 minutes at room temperature. After supernatant was removed, the pellet was resuspended in 1ml of freezing media (3T3-L1, L6 and HepG2: 10% DMSO, 70% DMEM, 20% FCS or NBCS / J774.2: 10% DMSO, 50% RPMI, 40% heat inactivated FCS) and transferred to sterile cryotubes before being frozen in cryogenic freezing containers (Nalgene cat. No. 5100-0001) at -70°C, allowing the cooling rate of 1 °C per minute. Cryotubes were transferred to liquid nitrogen storage tank after 48 hours.

2.2.1.5 Stimulation of cells

J774.2 macrophages were washed once in PBS then serum starved overnight in RPMI media without FCS containing 0.2% fatty acid free BSA. This was replaced with fresh RPMI/BSA media before stimulation

2.2.2 Cell lysate preparation

2.2.2.1 RIPA buffer

Cell monolayers were washed once with ice-cold calcium and magnesium free PBS. After removal of PBS, cells were lysed in RIPA buffer (0.1M Tris pH 7.4, 50mM NaCl, 50mM NaF, 5mM EDTA, 1% Tx-100, 0.1% SDS, 0.5% Deoxycholate) supplemented with 2μg/ml aprotinin, 1μM pepstatin, 10μM leupeptin, 50μg/ml ALLN and 1mM AEBSF . 500μl of lysis buffer was used per 10cm dish. Insoluble fraction was removed from lystates by centrifugation at 14,000 RPM for 10min at 4 °C . To avoid freeze-thawing cycles, lysates were aliquoted into 3 x 125μl aliquots before being stored at -70 °C.

2.2.2.2 Nuclear Extract Preparation

Cells monolayers were scraped in Hank's balanced salt solution supplemented with 0.1% fatty acid free BSA and collected by centrifugation at 1200 RPM for 5 minute at 4°c. Supernatant was removed and the pellet resuspended in 1ml ice-cold PBS. Cells were pelleted by centrifugation at 1200 rpm for 5minutes at 4 °C. Cell pellets were resuspended in 450μl Hypotonic buffer (10mM tris pH 7.6, 10mM NaCl, 3mM $MgCl_2$) supplemented with 2μg/ml aprotinin, 1μM pepstatin, 10μM leupeptin, 50μg/ml ALLN and 1mM AEBSF and 50μl 5% NP-50 and nuclei pelleted by centrifugation at 1200 RPM . The supernatant (post-nuclear supernatant) was stored at -70°C until analysis. Nuclei were washed in 1ml Hypotonic buffer before being resuspended in Dental&Latchman buffer (5mM Hepes pH 7.9, 26% glycerol, 1.5 $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) supplemented with 2μg/ml aprotinin, 1μM pepstatin, 10μM leupeptin, 50μg/ml ALLN and 1mM AEBSF. Then 12.5μl of 5mM NaCl was added. Lysate was rotated for 20 minutes at 4°C before the insoluble fraction was removed by centrifugation at 14,000 RPM for 20minutes at 4°C. The supernatant (nuclear extract) was stored at -70°C until analysis.

2.2.2.3 Homogenisation of Soleus muscle

Muscles were kindly isolated and treated by Dr. Jensen and stored at -70 °C before the extraction. Lysis was performed on ice. Briefly, 100μl of homogenising buffer (1% triton X-100, 50mM Hepes, 10mM EDTA, 2.5 mM Benzamidine, 0.1% SDS, 10mM $Na_4PO_2O_7$) supplemented with 2μg/ml aprotinin, 1μM pepstatin, 10μM leupeptin, 50μg/ml ALLN and 1mM AEBSF was added. Muscles were lysed with handheld plastic homogeniser on ice. After the tissue disintegrated, total volume was made up to 500μl and samples were incubated on a rotor at 4 °C for 1 hour. After the incubation insoluble fractions were

removed by centrifugation at 14000 RPM for 10 minutes at 4 °C. Id2 was immunoprecipitated from the samples as described below.

2.2.2.4 Lysis of cardiomyocytes

Primary rat cardiomyocytes were kindly isolated by Dr. Hillary, UCL. Tissues were lysed with 500µl Sucrose buffer and sonicated for 3 seconds and rest for 7 seconds. The cycle was repeated 7 times. Protein concentration was normalised and Id2 immunoprecipitated as mentioned below.

2.2.3 Protein analysis

2.2.3.1 Measurement of protein concentration with Bicinchoninic Acid (BCA) Calorimetric Protein Assay

BCA protein assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with calorimetric detection of the cuprous cation (Cu^{1+}) using reagent containing bicinchoninic acid (BCA). The purple colour is formed by chelation of two molecules of BCA with one Cu^{1+} ion. The complex shows strong absorbance at 562 nm that is nearly linear with increasing protein concentration over the working range of 20-2000 µg/ml. The procedure was carried out in 96 well flat bottom microplates. Briefly, diluted serum albumin was used to construct a standard curve. The original solution of 2mg/ml was diluted in PBS to form 50, 100, 150, 200, 250 µg/ml solution. 10µl of diluted serum albumin were used per well. A duplicate was used per standard concentration. The zero point was represented by PBS alone. A small proportion (10µl) of cell lysates from each sample was diluted 1:50 in PBS to ensure that protein concentration fall within the standard curve. Samples were vortexed thoroughly before 10µl was removed . The diluted samples were vortexed to mix thoroughly and 10µl was placed in 3 separate wells. Altogether, a triplicate reading was taken per sample. To prepare BCA assay reagent, reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) was mixed with reagent B (4% cupric sulfate) at ratio of 50: 1. After both solutions thoroughly mixed , 200µl of the working solution was added per assay well. The microplate was covered and incubated at 37 °C for 30 minutes. Absorbance was measured at 562nm using a plate reader.

2.2.3.2 Immunoprecipitation of proteins

For immunoprecipitation of Id2, cells lysates were prepared in Sucrose buffer (Wilson, 2001 #1232) (0.3M sucrose, 10mM Tris HCl pH8, 10mM NaCl, 3mM MgCl2, 0.5% NP-40) supplemented with 2µg/ml aprotinin, 1µM pepstatin, 10µM leupeptin, 50µg/ml ALLN and

1mM AEBSF). Protein concentration of each samples were measured with BCA assay and 500μg of total protein per sample was used per immunoprecipitation assay. The total volume of lysate was equalised with lysis buffer. Immunoprecipitation were performed using Id2 antibody diluted 1:100. Lysates were incubated with the antibody on a rotor for at least 2 hour at 4°C followed by addition of Protein-G agarose beads (30μl) which had been washed 3 times in lysis buffer to remove glycerol coating. The mixtures were incubated at 4°C for 1hour under rotation. Immune complexes formed were washed three times with lysis buffer before addition of sample loading buffer. Prepared sample was heated at 98 °C for 10 minutes before being centrifuged at 5000rpm for 30 seconds at 4°C.

2.2.3.3 SDS polyacrylamide gel electrophoresis

To visualize protein SDS PAGE was performed as described in Maniatis et al (p18.47-18.54). Vertical slab gels were prepared using either Hoefer or Biorad II or III casting apparatus. Whole cell lysate or immunoprecipitated proteins were separated by SDS PAGE gels (8-15% separating gel, 4% stacking gel) using Hoefer electrophoresis apparatus or Biorad PROTEAN III system. For cell lysate the appropriate amount of western blot loading buffer was added (20% glycerol, 4% SDS, 200mM DTT, 0.2M Tris buffer pH 6.8). Immunoprecipitates were washed 3 times with lysis buffer before addition of 30μl 2x western loading buffer. The samples were then boiled for 10 minutes before loading. Running buffer contains 3.03g/l Tris Base, 14.42g/l Glycine and 1g/l SDS. Electrophoresis was performed under constant voltage of 60V overnight for Hoefer or 150V for 90 minutes for Biorad PROTEAN system.

2.2.3.4 Wet transfer and western blotting

Samples on polyacrylamide gels were transferred onto Immobilon-P polyvinylidene fluoride (PVDF) blotting membrane (Millipore) using Biorad trans-blot cells. The membrane was rehydrated by soaking with methanol. The stacking gel was removed and transfer stack assembled then immersed in transfer buffer containing 0.05MTris base, 2.5M Glycine, 0.05% SDS and 20% methanol. The order of assembly were as followed.

- Sponge
- Filter paper
- PVDF membrane
- Gels
- Filter paper
- Sponge

Gel holders were placed in transfer tank set at 100V for 1 hour or 45 minutes for Id2. Membranes were then removed and block in blotto containing either 5% (x/v) fat-free dried

skimmed milk in TBS-T (0.1% v/v) (or 5% BSA in TBS-T for phospho-antibodies) for 1 hour at room temperature to saturate the binding sites on the membrane. The membranes were then incubated with the primary antibody overnight at 4 °C.

The primary antibody was then added at an appropriate dilution in either 1% milk TBS-T, 5% milk TBS-T or 5% BSA-TBS-T according to the manufacturer's recommendation and incubated overnight on a rocking platform at 4°C. The membrane was then washed three times with TBS-T for 5 minutes each and a suitable secondary antibody conjugated to horseradish peroxidase (HRP) at 1:2000 dilution in 1% milk or BSA TBS-T was incubated with the membrane for 45 minutes at room temperature. The membrane was then washed 3 times in TBS-T for 5 minutes each and antibody-antigen complex detected by Enhanced Chemiluminescence (ECL) (KPL) according to the manufacturer's instruction. Images were detected by Fuji LAS-1000 luminescent image analyser and analysed with Fuji Image Gauge software.

2.2.3.5 Detection of O-linked glycosylation by Western blotting

Cells were lysed in glycosylation-specific buffer containing (1% NP-40, 15mM Tris HCl pH 7.4, 150mM NaCl, 1mM EDTA) supplemented with 10μM O-(2-acetamido-2-deoxy-D-glucopyranosylidene amino N-phenylcarbamate) (PUGNAC), 2μg/ml aprotinin, 1μM pepstatin, 10μM leupeptin, 50μg/ml ALLN and 1mM AEBSF. Electrophoresis and western blotting were carried out as mentioned above. Membranes were blocked for 1 hour at room temperature with 10% BSA solution before being incubated with CTD 110.6 antibody against o-linked glycosylated residue for 1 hour at room temperature. Membranes were washed for 5 minutes 6 times with PBS-T before further incubation with secondary antibody goat antimouse IgM HRP for 1 hour at room temperature. Membranes were washed for 5 minutes 6 times before signals were detected by chemiluminescence with SuperSignal West Dura Substrate.

2.2.3.6 Strip membrane

To remove primary antibody and secondary antibody, membranes were incubated with 0.5mM NaOH for 10 minutes at room temperature on a platform shaker. Subsequently membranes were washed three times with TBS-T.

2.2.4 Nucleic acid manipulation

2.2.4.1 RNA extraction

RNA was extracted by protocol from the Sanger centre. The process was performed under safety fume hood. Briefly, 1ml of TRIZOL agent was added to 10 cm^2 petri dish. Cell monolayers were scraped by plastic cell scraper and lysate transferred to 2ml eppendorf tube. Lysates were incubated at room temperature for 5 minute before 200µl of chloroform was added per 1ml of Trizol reagent. Lysates were mixed vigorously by inversion for 15 seconds and incubated at room temperature for further 3 minutes. Samples were then centrifuged at 12000 x g for 15 minutes at 4 °C. The aqueous upper phase was transferred to a new 2ml eppendorf tube and 500µl of isopropanol added per 1ml of trizol used. Samples were incubated at room temperature for 10 minute then centrifuged at 12 000 xg for 15 minutes at 4 °C to pellet the RNA. Supernatants were removed and pellets washed with 1ml of 75% ethanol. Samples were vortexed and centrifuged at 7 500 xg for 5 minutes at 4 °C. Supernatant was removed and pellet air dried before being resuspended in 50µl of DEPC water and incubated at 60 °C until pellet was dissolved completely.

2.2.4.2 Reverse transcription

To reverse transcribe RNA into first strand DNA, 0.5 µg oligo dTs was mixed with 5µg RNA and the volume adjusted to 12 µl with sterile autoclaved water. The mixture was heated to 70 °C for 10 minutes and chilled immediately on ice. The tubes were then centrifuged briefly to collect the content at the bottom. Subsequently, 4µl first strand buffer (250mM Tris HCl pH 8.3, 375 mM KCl, 15mM $MgCl_2$), 2µl of 0.1M DTT and 1µl of dNTP mix were added. The content was mixed gently and incubated at 42 °C for 2 minutes. After the incubation, 1µl of SUPERSCRIPT II was added and the mixture incubated at room temperature for 10 minutes before further incubation at 42 °C for 50 minutes. Subsequently the reaction was inactivated by heating at 70 °C for 15 minutes.

2.2.4.3 Polymerase chain reaction

PCR amplifications were performed in 50µl reaction volume containing 1x cloned PfuTurbo DNA polymerase reaction buffer (20mM Tris-HCl, pH 8.8, 2mM MgSO4, 10mM KCl, 10mM (NH4)2SO4, 0.1% Triton X-100, 1mg/ml nuclease free BSA), 200µM each dNTP, 10ng template DNA, 20pmol primers and 2.5U Pfu Turbo polymerase all purchased from Strategene. Amplification was performed in a Techno Progene thermocycler. Cycling parameters were as followed: A single 2 minutes denaturation step at 95 °C followed by 30

cycles of 30s denaturation at 95 °C, 30s annealing at 5 °C below the lowest primer melting temperature and 1min per kb of the target extension at 72 °C.

2.2.4.4 Electrophoration of DNA fragments

Agarose (gibco/BRL) was dissolved in TAE buffer (40mM Tris acetate, 1mM EDTA by heating cooled and ethidium bromide added at a concentration of 1μg/ml before gel casting. The percentage agarose used was 1%. 6x gel loading buffer (0.25% bromophenol blue, 30% glycerol dissolved in water) was added to the DNA samples which were electrophoresed in TAE buffer at 60mA. For the determination of DNA size 1kb DNA mass ladder was electrophoresed simultaneously alongside. DNA bands were visualised by illuminating the gel on a UV light box.

2.2.4.5 Transformation

Competent cells were purchased from Strategene. XL-1 Blue competent cells were used for plasmid preparation. 100ng of plasmid was mixed with 100μl of freshly thawed competent cells and heat shocked at 42 °C for 90 seconds followed by 90 seconds incubation on ice. Cells were allowed to recover by the addition of 1ml of LB media without antibiotics and then shaken for 1 hour at 37 °C before being plated on agar plate containing 100μg/ml ampicillin followed by overnight incubation at 37 °C.

2.2.4.6 Propagation and purification of plasmid DNA

A 5ml starter culture was innoculated with a single colony isolated from a freshly streaked agar plate and incubated at 37 degree on a shaker for 8 hour. The starting culture was diluted into 100ml LB media at 1: 500 dilution. After an overnight incubation at 37 °C with shaking, cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4 °C. Pelleted cells were further processed using a QIAfilter Plasmid Maxi Kit (Qiagen) according to the manufacturer's instruction. Briefly, cells were resuspended in 10ml buffer P1. After the clumps had disappeared, buffer P2 was added and container inverted 4-6 times before the mixture was incubated for 5 minutes at room temperature. During incubation QIAfilter cartridge was prepared. After 5 minutes 10ml of buffer P3 was added to the lysate and the container inverted gently. Mixture was transferred to the cartridge directly and incubated for 10 minutes at room temperature. During this period, QIAGENtip was equilibrated with buffer QBT. After incubation, mixture from QIAfilter was filtered into the QIAGENtip. Clear lysate was allowed to enter resin by gravity flow. After lysates were filtered, QIAGENtip was washed with 30ml buffer QC twice before DNA was eluded into glass tube by adding 15 ml of buffer QF. DNA was precipitated by addition of 10.5 ml room temperature isopropanol and centrifuged immediately at 15, 000 x g for 30 minutes to

precipitate DNA pellet. Supernatant was removed and DNA pellet washed with 70% ethanol and transferred to Eppendorf tube. Tube was centrifuged at 14,000 RPM to pellet DNA, ethanol was removed and DNA pellet air dried for 30 minutes before being dissolved in TE buffer (10mM Tris pH8 and 1mM EDTA pH 8) pH8.

2.2.4.7 Determination of DNA concentration and purity

DNA concentration was determined spectrophotometrically by measuring OD at 260nM. Concentration was then calculated with the following equation (OD260=1=50μg DNA) (Reading at OD 260 x 50 x 200) / 1000to give a final value in μg/μl. To determine plasmid preparation quality a ratio of OD260 raw reading/OD280 raw reading was used . A good preparation should give a ratio between 1.8 to 2. Ratio of every preparation used in this experiment falls within the optimum ratio.

2.2.5 Transfection

2.2.5.1 Transfection of J774.2 with Fugene 6 transfection reagent

One 75 cm^2 flask of J774.2 cells was passaged into 4 x 6 cm diameter dishes in antibiotic-free RPMI 1640 media supplemented with 10% serum 48 hours before the start of transfection procedure. Cells were refed after 24 hours and fresh media was added before transfection. The ratio of Fugene:Optimem:DNA used in this transfection is 1.2μl:38.8μl:0.4μg respectively. Master mix of Fugene was added to Optimem. It was vital to prevent contact between Fugene solution and plastic tubing. Optimem was added to the Eppendorf tube before addition of Fugene. The mixtures were incubated for 5 minutes at room temperature. The required amount of plasmid DNA was prepared in separate Eppendorf tubes. The master mix of Fugene 6 and Optimem was added to plasmid DNA and mixed by pipetting gently up and down. The mixture was incubated at room temperature for 15 minutes before being added to the cell culture media. Cells were incubated with transfection mixture for 24 hours before further treatment.

2.2.5.2 Optimisation of transfection in J774.2 cells

J774.2 cells were notoriously difficult to transfect. We attempted transfection with JetPEI Man, Gene Porter and Lipofectamine Plus before deciding that Fugene 6 gave the highest transfection efficiency with the least cellular toxicity . To assay for the best transfection condition in J774.2 cells, cells were first transfected with positive control pGL3 promotor enhancer plasmid or negative control cGL3 basic plasmid together with internal positive control renilla pTK. The ratio of firefly to renilla was 10:1. To assay the optimum amount of

DNA, cells were transfected with 2.5 μg DNA, 5μg DNA, 8μg DNA. As shown in figure 1, Fugene 6 with 2.5 μg DNA gave the optimum reading with the least amount of DNA required.

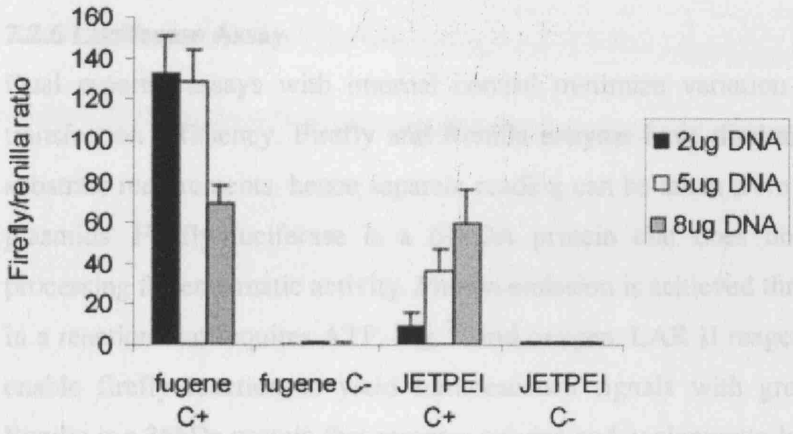


Figure 2.1 Comparison of transfection efficiency

To assess optimal transfection time, reading from positive control and pGL3 enhancer (HSL promotor) under 10% serum was taken after 24 hours and 48 hours. We found no difference between 24 hour and 48 hour time point, hence treatment of J774.2 commenced after 24 hours.

2.2.5.3 Transfection of CHO-IR cells (CHO cells stably expressing insulin receptor) with lipofectamine reagent

Around 24 hours before transfection CHO-IR cells were passaged in antibiotic free F12 media supplemented with 10% serum at 1:6 ratio to form 50% confluent culture on 6 wells plate. After 24 hours cells monolayer were washed once with sterile PBS before 200 μl Optimem was added per well. Lipofectamine was used as standard reagent for CHO-IR cells, the optimum ratio was 2μg plasmid per 5μl lipofectamine. Firstly, 2μg of total DNA was prepared. In this experiment, 0.5 μg pGL3 basic (HSL promoter), 0.1 μg pCMV SREBP-1, 0.5 μg pCMV Id2 and 0.02 μg renilla pTK was used when required. The total amount of DNA was adjusted to 2μg with empty pcDNA3. An equal amount of DNA was used for each transfection regardless of plasmid composition. DNA was diluted in 25 μl Optimem and mixed gently. For 2μg DNA, 5μl Lipofectamine was diluted in 25 μl Optimem in a separate tube. Subsequently Lipofectamine + Optimem solution and DNA + Optimem solution were combined at incubated at room temperature 30 minutes to allow DNA liposome complex to form. After 30 minutes, 150μl of Optimem was added to the complex and the complex mixed gently before it was overlayed on the cell monolayer. Cells were incubated with

transfection reagent for 6 hour at 37 °C before transfection media was replaced with F12 media containing 10% serum for 24 hours. After 24 hours, cells were lysed and luciferase assay performed.

2.2.6 Luciferase Assay

Dual reporter assays with internal control minimize variation due to cell viability and transfection efficiency. Firefly and Renilla enzyme have dissimilar enzyme structures and substrate requirements, hence separate reading can be taken from cells transfected with both plasmids. Firefly luciferase is a 61kDA protein that does not require posttranslational processing for enzymatic activity. Photon emission is achieved through oxidation of luciferin in a reaction that requires ATP, Mg^{2+} and oxygen. LAR II reagent contains coenzyme A to enable firefly reaction to yield luminescence signals with greater intensity and length. Renilla is a 36kDa protein that requires oxygen and coelenterate-luciferin as substrate.

2.2.6.1 Cell lysis for luciferase assay

Luciferase assay kit was purchased from Promega. Samples to be assayed for luciferase activity was lysed in a specific lysis buffer (PLB) provided with the kit. PLB was supplied as a 5x concentrated solution. The concentrated solution was diluted to a 1x working solution with distilled water and cooled on ice. Subsequent to lysis buffer preparation, cell medium was transferred to a 15 ml Falcon tube. Cell monolayer was scraped in 5 ml PBS without Calcium or Magnesium and the suspension transferred to the same Falcon tube. Cells were pelleted by centrifugation at 1200 RPM for 5 minutes at 4 °c. After the supernatant was discarded, cells were resuspended in 100µl PLB lysis buffer and the suspension transferred to a 2 ml eppendorf tube. Each tube was vortexed for 10 seconds before the samples were stored at -70°C until analysis.

2.2.6.2 Preparation of reagent and equipment for luciferase assay

To prepare LARII reagent, lyophilized luciferase assay substrate was resuspended in 10ml of the supplied luciferase assay buffer II. Working solution was stored at –70°C in small aliquots. To prepare Stop and Glo reagent, the 50x substrate solution was diluted with Stop & Glo buffer in a glass tube just before use. The luminometer was programmed to take 10 seconds measurement period with a 2 second delay.

2.2.6.3 Dual luciferase reporter assay

To perform luciferase assays, 100µl of working solution of LARII was placed in a disposable luciferase cuvette before the addition of 20µl of assay sample. Sample and LARII reagent were mixed by pipetting action 3 to 4 times. The cuvette was placed in the luminometer

cavity and the firefly luciferase activity measured. Subsequently, the cuvette was removed and 100µl of working solution of stop&glo reagent added to the mix to stop firefly activity and to simultaneously activate reading for renilla activity. The mixture was vortexed briefly for 2 seconds before the cuvette was returned to the luminometer and renilla luciferase measurement taken. The final reading was expressed as a ratio between firefly and renilla luciferase activity.

Chapter 3 Results

Effects of Glucose on Transcription factors and Hormone-Sensitive Lipase (HSL) in Macrophages

3.1 Abstract

It has previously been found that a reduction in the protein level of Hormone Sensitive Lipase (HSL) was associated with increased cholesterol ester accumulation in macrophages. Previously O.Rourke et al showed that at 5mM glucose, chronic leptin treatment increased HSL expression and activity. The opposite was observed at 20mM glucose. When cells were pre-treated with high glucose, chronic treatment with insulin or leptin decreased HSL expression after 24 hours. This chapter investigates how changes in levels of transcription factors might be associated with this decrease. We found that pre-treatment of J774.2 macrophages with 20 mM glucose lowered the response of the HSL promoter to insulin and leptin at the 24 hour time point. Analysis of the promoter sequence revealed putative binding sites for PPAR gamma and SREBP protein. We therefore investigated how glucose altered levels of these transcription factors. We found that PPAR gamma expression was lowered by high glucose at 6 hours and 24 hours. There was no change in response to leptin. Levels of the mature SREBP-1 increased with leptin at 5mM glucose, leptin in combination with insulin at 5mM glucose and high glucose alone. On the other hand, leptin in combination with high glucose as well as leptin and insulin combined with high glucose decreased SREBP-1 mature form. The pattern of HSL expression broadly matched the effects on SREBP-1 and together with the presence of numerous SRE sites on HSL promoter this suggested that SREBP-1 could be the mediator of the glucose effects on HSL expression. In support of this we found that co-expression of SREBP-1 with HSL promoter luciferase constructs increased the promoter activity. We concluded that reduction in level of mature SREBP-1 when cells were pre-treated with 20mM glucose may lead to reduction in HSL promoter activity in response to insulin and leptin.

3.2 Introduction

Atherosclerotic lesion development is preceded by penetration of blood borne mononuclear cells into the intima of blood vessel walls. These monocytes subsequently differentiate into macrophages, which can take up lipids and develop into foam cells in response to cytokines, chemokines and modified LDL in the environment. These lipid-filled macrophages are called foam cells and are an early and persistent feature of fatty streak lesion and are pivotal in lesion development.

Pathological conditions associated with metabolic syndrome such as hyperinsulinaemia and hyperleptinaemia have been linked to proatherogenic changes in macrophage gene expression and accelerated lesion development (Fonseca 2000; Saito 2000). Most of their effects have been attributed to enhancement of transmigration, oxidation of LDL, up regulation of modified LDL receptors and increased production of proinflammatory cytokines. Recent work from this lab has provided evidence to support the hypothesis both hormones directly affect intracellular cholesterol ester levels in macrophages by downregulating the expression of HSL when macrophages are pre-treated with high glucose (O'Rourke 2002).

HSL hydrolyses ester bond in triacylglycerol and cholesterol ester. It is responsible for triglyceride hydrolysis in adipose tissue, muscle, heart and pancreatic beta cells. The same enzyme catalyses cholesterol ester hydrolysis in adrenals gland, testes and ovaries {Yeaman, 2004 #625}. Evidence suggests that HSL is the enzyme responsible for neutral cholesterol ester hydrolase activity in macrophages. This is based mainly on the disappearance of neutral cholesterol ester hydrolase activity in J774.2 and Raw macrophages when HSL is immunoprecipitated from cell extract (Small, Goodacre et al. 1989). Subsequently several groups confirmed this association. The presence of mRNA for HSL has been demonstrated by RT-PCR (Khoo 1993). Overexpression of HSL in THP-1 macrophages by adenovirus-mediated gene delivery removes cholesterol ester from cells preloaded with acetylated-LDL completely and Raw 264.7 cells stably over expressing HSL exhibit 2-3 fold increase in nCEH activity when loaded with lipid (Escary 1998; Okazaki 2002). Expression of HSL mRNA in human and mouse monocyte/macrophages has been detected with PCR and northern blotting (Khoo 1993; Reue 1997). Finally, another experiment *in vivo* reports reduced level of HSL expression in human macrophage foam cells. The phenomenon is accompanied by low nCEH activity and resistance to HDL-mediated cholesterol efflux (Harte 2000).

HSL is regulated acutely by phosphorylation and translocation. HSL is a serine hydrolase with active serine (423 in rat and 424 in human) located in the characteristic Gly-Xaa-Ser-Xaa-Gly motif found in lipases and esterases. Site-specific mutagenesis shows that Asp (703 in rat and 693 in human) and His (733 in rat and 723 in human) are the other members of the catalytic triad (Holm, Davis et al. 1994; Contreras, Karlsson et al. 1996; Osterlund, Danielsson et al. 1996; Osterlund, Contreras et al. 1997). Regulation of HSL has been studied most extensively in adipocytes where it is well established as a rate-limiting enzyme of triacylglycerol (TAG) lipolysis. HSL is one of the enzymes determining whole body fuel availability and it accounts for most of lipolysis in fatty tissues in the post-absorptive state (Li, Sumida et al. 1994). Adrenergic agents stimulate the conversion of TAG by HSL into free fatty acid and glycerol for metabolism.

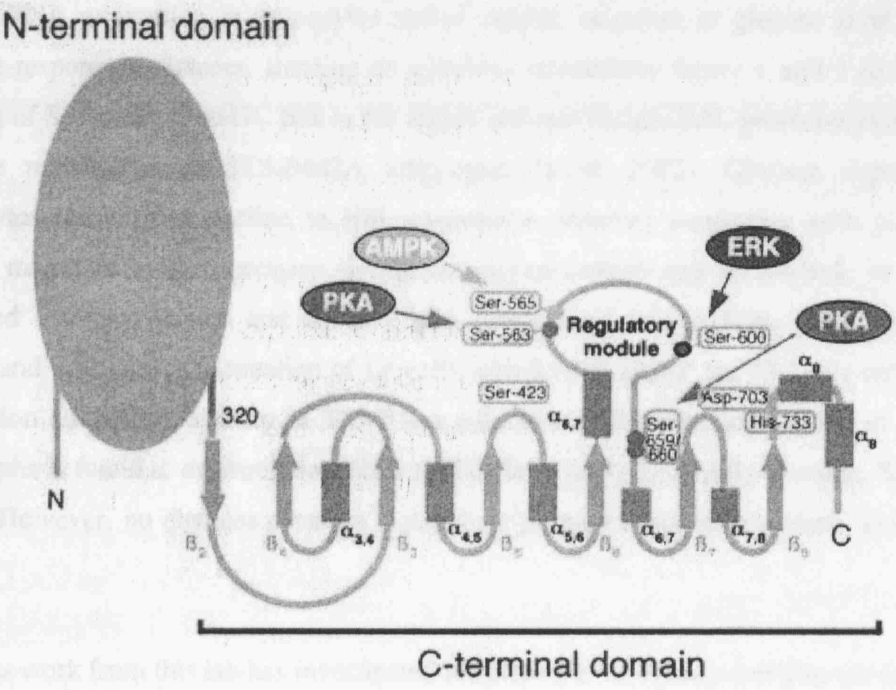


Figure 3.1 Schematic structure of rat HSL (Kraemer and Shen 2002). The N-terminal 320 amino acids are depicted as a globular structure since its precise structure cannot currently be modelled. The C-terminal portion forms alpha/beta hydrolase structure and contains the catalytic triad: Ser-423, Asp 703, His-733. The regulatory module in the C-terminus contains regulatory phosphorylation sites.

Conversely, insulin downregulates HSL activity to promote TAG storage. Acute regulation of HSL activity occurs through post-translational modification of the regulatory loop on its C-terminal domain. Adrenergic signals stimulate G-protein-coupled adenylate cyclase

activity. This raises intracellular cAMP levels and activates PKA. PKA then phosphorylates HSL on serine 563, 659 and 660. These phosphorylations increase HSL activity (Egan, Greenberg et al. 1992; Clifford, McCormick et al. 1997; Anthonsen 1998) and stimulate the translocation of HSL from the cytosol of 3T3-L1 adipocytes into the intracellular lipid droplets (Brasaemle, Levin et al. 2000). In fat cells, insulin downregulates HSL activity by stimulating the hydrolysis of cAMP by PDE3B (Degerman 1990).

While acute regulation of HSL activity occurs through post-translational mechanism, pretranslational controls regulate HSL activity and expression under chronic condition. There are no reports of post-transcriptional modification of HSL mRNA; however pre transcriptional control of HSL expression has been extensively studied in adipose tissues. HSL expression level differs between distinct fat storage depots. Subcutaneous fat in human is reported to have higher HSL mRNA than omental fat (Reynisdottir, Dauzats et al. 1997). HSL mRNA expression in adipocytes shows chronic response to glucose level through a glucose-responsive element. Binding of upstream stimulatory factor 1 and 2 to E box and binding of Sp1 and Sp3 to GC box in the region activate human HSL promoter in response to glucose metabolism in 3T3-F442A adipocytes (Smih 2002). Glucose deprivation in adipocytes results in a decline in HSL expression, whereas incubation with glucose and insulin maintains HSL expression during adipocytes culture and an increase in basal and activated lipolysis (Botion and Green 1999). Other regulators of HSL expression include cAMP and TNF-alpha. Incubation of fat cells with 8-CPT-cAMP for 12 hours reduces HSL expression and nCEH activity in 3T3-F44A adipocytes (Plee-Gautier, Grober et al. 1996). TNF alpha is found to downregulate HSL mRNA level in 3T3-L1 cells (Sumida, Sekiya et al. 1990). However, no changes occur in isolated rat primary adipocytes (Green, Dobias et al. 1994).

Previous work from this lab has investigated the effects of hormones and glucose on the HSL levels in macrophages (O'Rourke 2002). In low glucose condition, leptin was found to increase HSL expression while the reverse was observed in high glucose. To date, the mechanism and transcription factors mediating the effects have not been identified. Since changes in HSL expression in macrophages may underline accelerated lesion development in subjects with hyperinsulinaemia and hyperleptinaemia and hyperglycaemia, it is important to gain a more detailed understanding of the regulatory system controlling HSL expression in macrophages. This chapter describes studies aimed at identifying such mechanisms in J774.2 macrophages.

3.3 Results

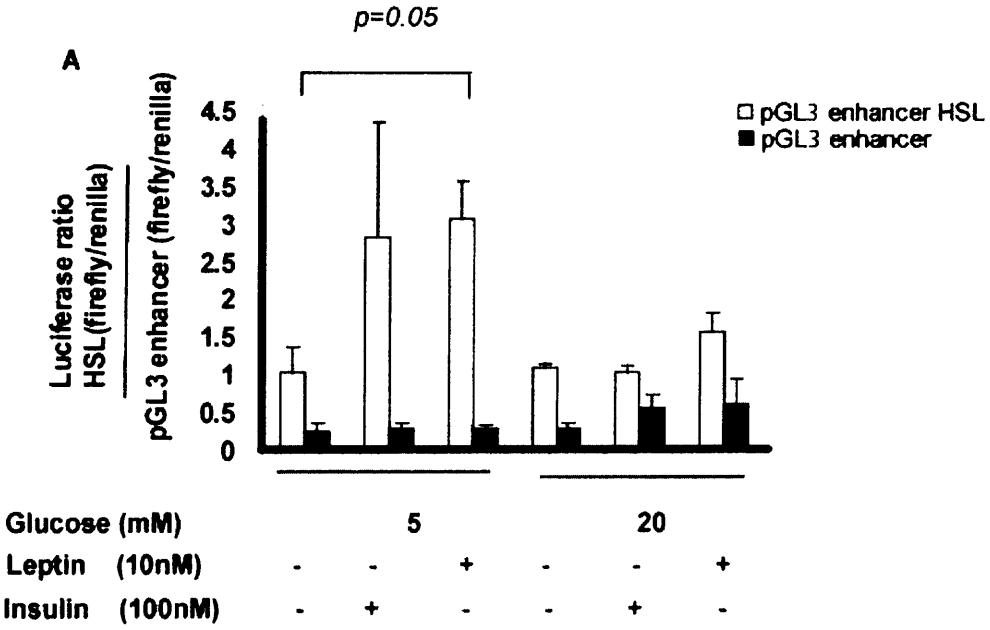
3.3.1 HSL promoter response in J774.2 macrophages. Pretreatment with 20mM glucose altered response of HSL promoter to chronic insulin and leptin treatment in J774.2 macrophages

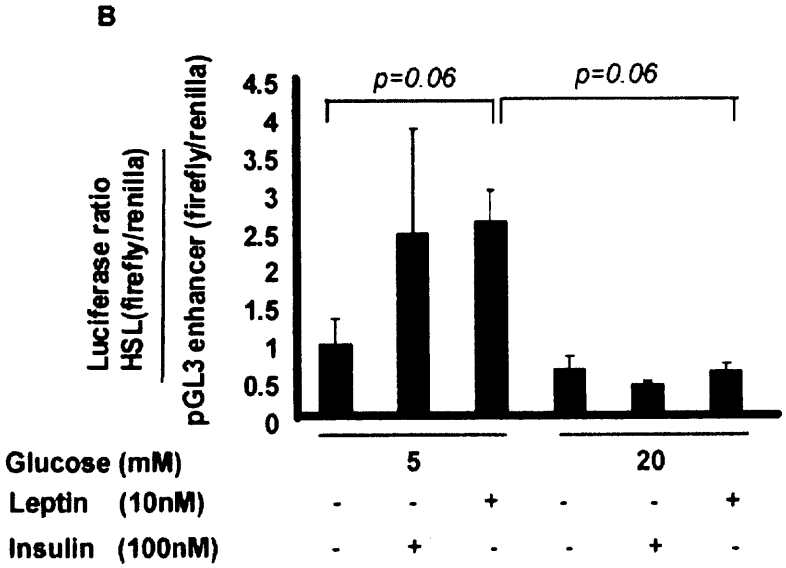
Work from this lab had previously shown that levels of HSL protein in J774.2 macrophages changes in response to glucose, insulin and leptin but it is not known if these changes occurred at transcriptional level. Therefore, we analysed whether glucose, insulin and leptin changes the activity of HSL promoter. A HSL promoter construct attached to luciferase reporter has been kindly provided by Dr. Talmud , University College London. The promoter fragment spans residue -86 to +85 bp upstream of the transcription start site on Human HSL promoter exon B. A sizeable part of HSL transcripts originate from this exon. Analysis of transcription binding sites revealed putative Sp-1 site, C/EBP site and several potential SRE sites (Talmud, Palmen et al. 1998). We transfected the construct into J774.2 macrophages, growing in antibiotic-free media supplemented with 10% serum, with the luciferase construct using Fugene 6 transfection reagent. Cells were incubated with transfection media for 24 hours. Subsequently they were starved in serum-free media containing 2% serum overnight in 5mM or 20mM glucose prior to stimulation with insulin or leptin for further 24 hours in 5mM or 20mM glucose. The 24 hour time point was chosen because the most profound effect occurred with HSL expression at this time point. After 24 hours cells were lysed and luciferase readings taken under the protocol provided.

As shown in Figure 3.2A, there was a significant increase in HSL promoter activity in response to leptin or insulin at 5mM glucose. However, the response to hormonal treatment was greatly lowered when cells were pre treated with 20mM glucose. Readings from control cells transfected with empty pGL3 enhancer plasmid increased when cells were treated with high glucose and insulin or high glucose and leptin. To take into account these changes, luciferase ratio from cells transfected with plasmid containing HSL promoter was normalised to the reading from cells transfected with pGL3 enhancer plasmid receiving similar treatments. The normalised result is shown in Figure 3.2B. After normalisation, we found a significant increase in HSL promoter activity in response to leptin at 5mM glucose. The readings from leptin treated cells grown in 5mM glucose media were approximately 4 folds higher than the readings from leptin-treated cells grown in 20mM glucose media (p value of 0.06).

Figure 3.2 HSL promoter activity changes when J774.2 macrophages were treated with a combination of glucose and leptin

J774.2 cells were grown in antibiotic-free media for 24 hours. Subsequently cells were transected with pGL3 enhancer plasmid containing -86 to +84 fragment of HSL promoter cloned into the Acc651-HindIII site and renilla plasmid using Fugene 6 transfection reagent in RPMI 1640 media containing 10% serum. After 24 hours culture media were changed to fresh RPMI 1640 media containing 2% serum and 2mM or 20mM glucose. After an overnight incubation cells were treated with 1μM insulin or 10nM leptin for 24 hours. Subsequently cells were lysed with luciferase lysis buffer and firefly/renilla ratio determined by luminometer. pGL3 enhancer-promoter was used as positive control for transfection. Treatments were performed simultaneously on cells transfected with pGL3 enhancer plasmid. Changes in firefly luciferase activity were observed with empty pGL3 enhancer. In figure A luciferase ratio from HSL (firefly/renilla) is represented separately from luciferase ratio from pGL3 enhancer (firefly/renilla). Readings are normalised to the basal HSL promoter reading at 5mM glucose. In figure B result is represented as the ratio of HSL (firefly/renilla) over pGL3 (firefly/renilla) for a particular treatment. Readings are normalised to basal HSL reading at 5mM glucose. Two separate experiments were performed in triplicate. Results represent quantification of a single experiment performed in triplicate.


A
p=0.05
Luciferase ratio
HSL(firefly/renilla)
pGL3 enhancer (firefly/renilla)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pGL3 enhancer HSL
pGL3 enhancer
Glucose (mM) 5 20
Leptin (10nM) - - + - - +
Insulin (100nM) - + - - + -

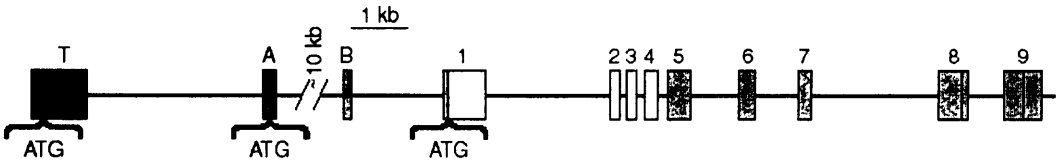

B
p=0.06
p=0.06
Luciferase ratio
HSL(firefly/renilla)
pGL3 enhancer (firefly/renilla)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Glucose (mM) 5 20
Leptin (10nM) - - + - - +
Insulin (100nM) - + - - + -

We conclude that high glucose levels attenuate the effects of insulin and leptin on the activity of HSL promoter. These changes closely match the changes in HSL protein levels that were previously observed in J774.2 cells treated in the same manner (O'Rourke 2002). Therefore this supports the concept that the effects of glucose are at least in part, mediated by changing the transcriptional regulation of HSL. Consequently, we attempted to identify transcription factors which contributed to the effects of glucose by analysing how glucose regulates transcription factors with potential binding sites on HSL promotor.

3.3.2 HSL gene structure

The 5'-untranslated region and HSL gene from mouse and human has been well characterised. The human HSL gene consists of two non-coding exons (exon A and exon B) upstream of 9 coding exons (exon one to exon nine). An additional exon T has been observed in human HSL transcript from testis. The mouse HSL gene contains 5 non-coding exons, denoted exon A to exon E and 9 coding exons. Mouse exon C is the equivalent of human exon B. Exon arrangement in human HSL gene is shown in Figure 3.3 (Yeaman 2004).

Figure 3.3 HSL gene structure



Promoter analysis in 3T3-L1 adipocytes shows that there are several transcriptional start sites. In mouse adipocytes, there are at least five alternative 5'-termini in HSL transcripts but splicing of pre-mRNA sequences occurs at the same position located 20nt upstream of translational initiation codon on exon1. Around 50-70% of HSL transcripts originate from exon C, 10% starts from exon A and around 5-30% starts from exon 1. All three forms are translated to a single protein product starting with amino acid decoded on exon 1 (Laurin 2000). The use of alternative promoters may underlie tissue specific expression. Depending on the relative concentration of transcription factors in a particular tissue, different regulatory elements are preferentially activated, leading to the generation of mRNA of varied length, stability and translational efficiency. Activation of alternative promoters and tissue specific multiplicity of mRNA species with different 5-end termini may underline the opposing effects of glucose in combination with insulin or leptin on HSL expression in 3T3-

L1 adipocytes and J774.2 macrophages. At present, the relative abundance of HSL mRNA species in macrophages has not been analysed.

A region of high promoter activity upstream of human exon B contains many conserved blocks containing potential binding motifs for regulatory elements. A sequence alignment marked with potential transcription factors binding sites is shown below (Laurin 2000). Figure 3.4 represents sequence upstream of exon B and Figure 3.5 represents sequence upstream of exon A. Exonic sequences are shown in upper case and intronic sequences in lower case. Position 1 corresponds to nucleotide number 1 of exon C. In addition to sites labelled on the diagram, the human HSL promoter also contain 3 more SRE sites on the minus strand at -632, -607, -78, 50 and 1 more SRE site on the + strand at -58 relative to exon B start site (Talmud 1998). This together with numerous SRE sites on mouse promoter suggests that SREBP could contribute to the regulation of HSL expression. The involvement of SREBP would link changes in HSL level to intracellular cholesterol and lipid concentration.

The case for SREBP is further strengthened by sensitivity of HSL expression to cellular sterol levels. In 3T3-L1 adipocytes, incubation of cells with cholesterol did not change N-CEH activity, but incubation with 25-hydroxycholesterol decreases the activity and the expression of HSL in a concentration-dependent manner (Miura, Nagura et al. 2003). Similar reduction in protein level of HSL also occurs upon incubation of J774.2 macrophages with 25-hydroxycholetserol or VLDL (Jepson 1996). These findings suggest that Sterol Regulatory Element Binding Protein (SREBP), itself inhibited by 25-hydroxycholesterol, could be involved in the regulation of HSL expression.

Figure 3.4 Upstream of exon B

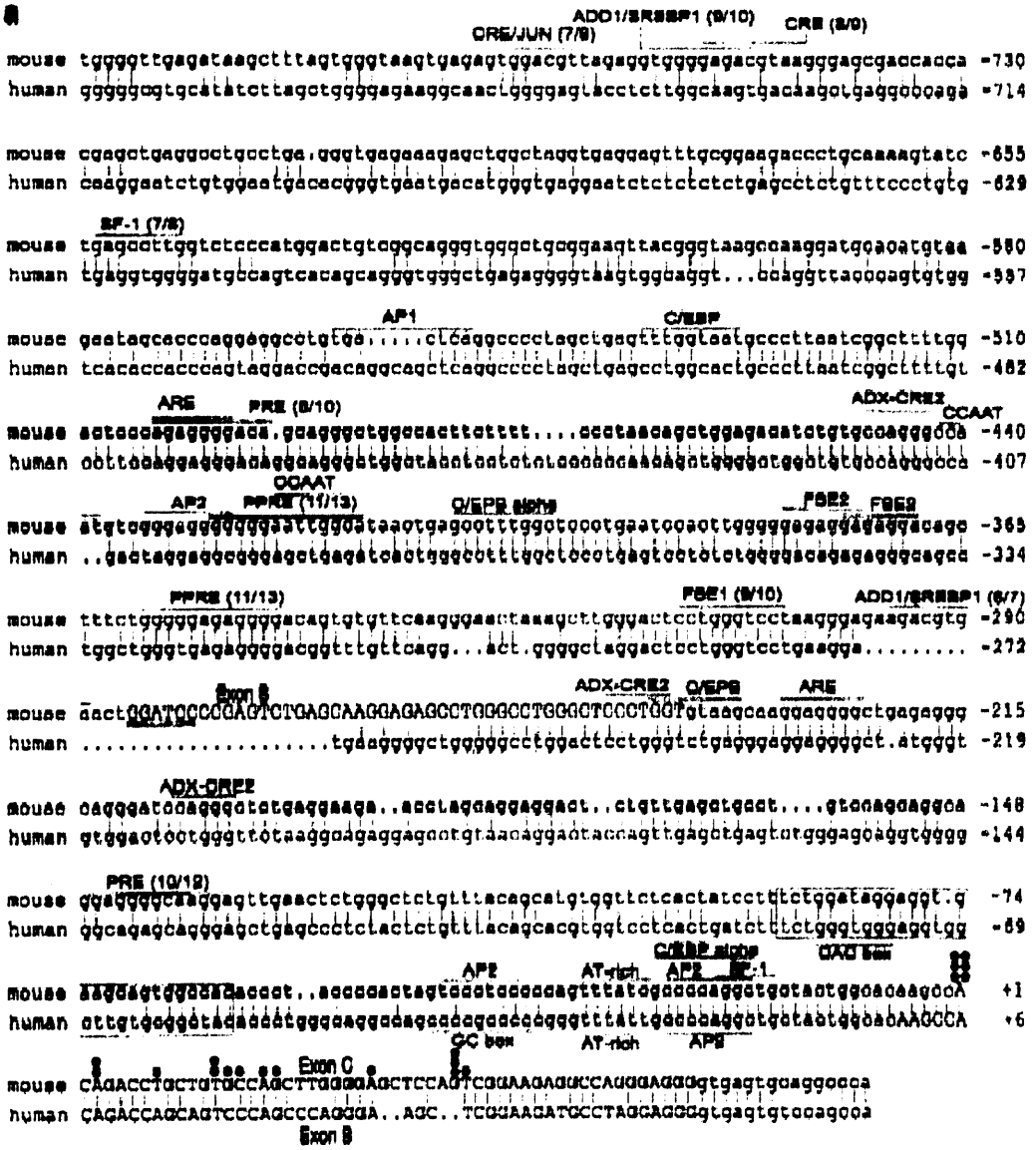
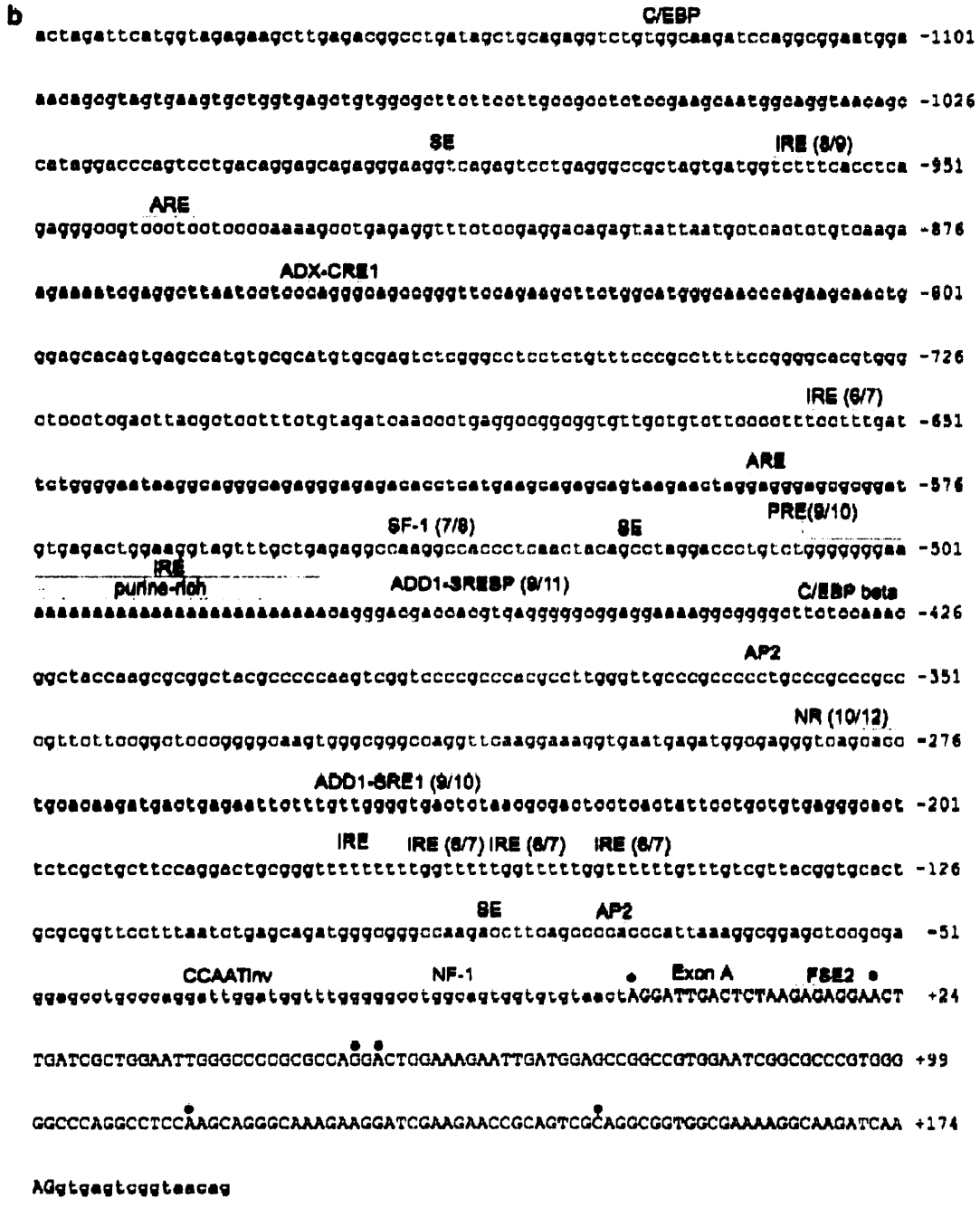


Figure 3.5 Upstream of exon A



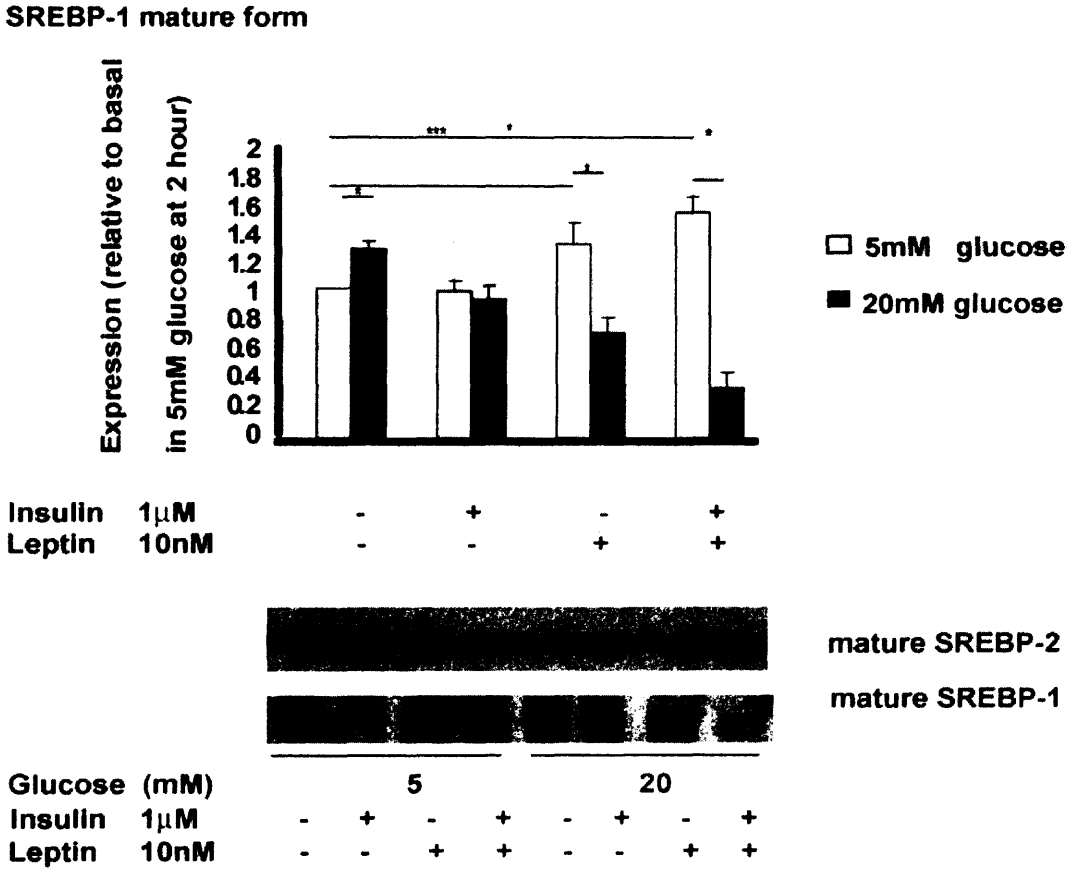
3.3.3 Changes in SREBP maturation in 20mM glucose

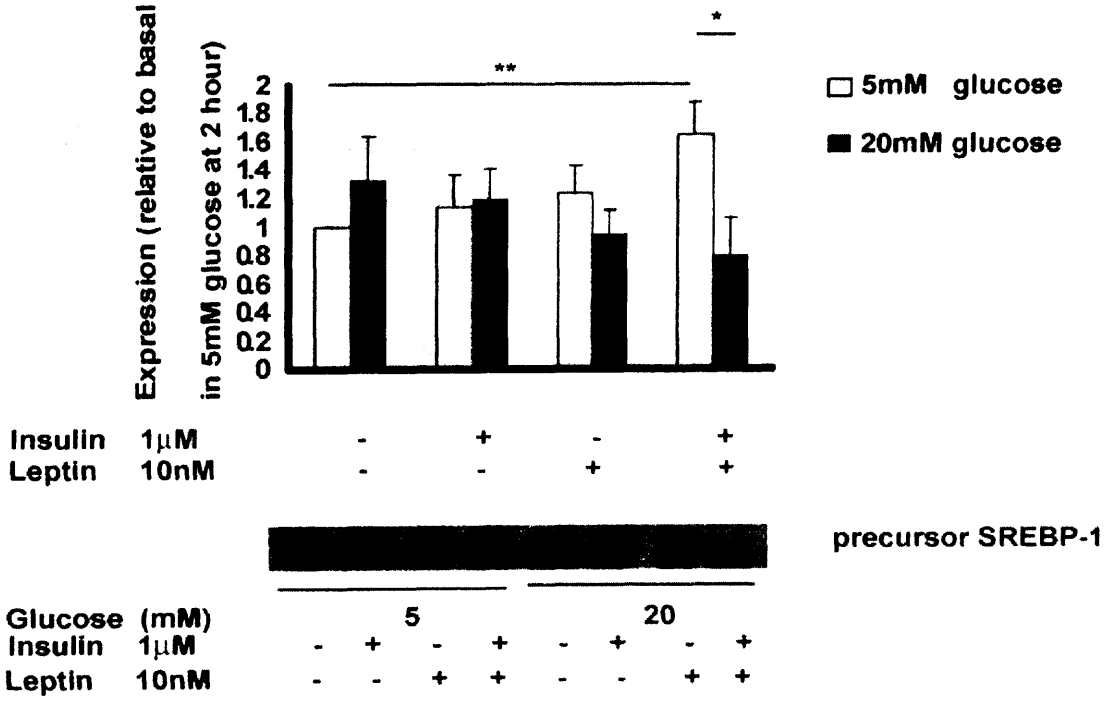
As summarised in the main introduction, SREBP must be cleaved to be active. Cleavage of SREBP is under the control of hormone, cholesterol concentration and fatty acid. Both SREBP-1 and SREBP-2 are sensitive to cleavage inhibition by 25-hydroxycholesterol but only SREBP-1 is sensitive to cleavage inhibition by fatty acid (Hasty 2000). The potency of inhibition by fatty acid increases with increasing chain length and degree of unsaturation with the reduction occurring at both mRNA level and during cleavage process (Hannah, Ou et al. 2001). Cleavage of SREBP-1 also requires contributions from insulin, possibly through its effect on insig protein (Hegarty, Bobard et al. 2005). Insulin modulates SREBP cleavage via a PI3-kinase-dependent pathway (Azzout-Marniche, Becard et al. 2000).

To assess changes in SREBP level, J774.2 cells were pretreated with 5mM or 20mM glucose overnight before further treatment with insulin, leptin or a combination of insulin and leptin in 5mM or 20mM glucose. Cells were harvested at the designated time point and nuclear extract separated from post-nuclear supernatant. We stained and viewed nuclear preparations under the microscope to check for contamination and the presence of nuclei. Samples were separated on Polyacrylamide gel and probed with anti SREBP-1 antibody. As shown in Figure 3.6, at the 24 hour time point striking differences occurred with the mature form of SREBP-1. In 5mM glucose media, leptin treatment increased levels of the mature form of SREBP-1. There was little effect after insulin. Treating cells with 20mM glucose increased levels of SREBP-1 mature form. Interestingly, levels of mature form of SREBP-1 after leptin treatment in 5mM glucose was approximately 2-fold higher than levels of mature form of SREBP-1 after leptin treatment in 20mM glucose. There were no significant changes to the precursor form. A complimentary study showed no changes in levels of SREBP-2.

Figure 3.6 SREBP-1 level changes when J774.2 cells were treated with a combination of glucose, insulin and leptin

J774.2 cells were pre treated overnight in RPMI 1640 media supplemented with 2% serum before being treated for 24 hours with leptin, insulin or a combination of both insulin and leptin. Cells extracts were prepared with nuclear extraction protocol. Samples were separated into nuclear fraction and post nuclear supernatant. Equal amount of protein from each sample were separated by SDS-PAGE electrophoresis and SREBP-1 detected by western blot. Protein bands were visualised by chemiluminescence . Relative level of SREBP-1 was quantified. Data represent the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicates differences where $P \leq 0.01$, ** indicates $P \leq 0.05$ and *** indicates $P \leq 0.09$. Figure A represents the level of SREBP-1 mature form and figure B represents level of SREBP-1 precursor form.


SREBP-1 mature form
Expression (relative to basal in 5mM glucose at 2 hour)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5mM glucose
20mM glucose
Insulin 1μM - + - +
Leptin 10nM - - + +
mature SREBP-2
mature SREBP-1
Glucose (mM) 5 20
Insulin 1μM - + - + - + - +
Leptin 10nM - - + + - - + +


SREBP-1 precursor form
Expression (relative to basal in 5mM glucose at 2 hour)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5mM glucose
20mM glucose
Insulin 1μM - + - +
Leptin 10nM - - + +
precursor SREBP-1
Glucose (mM) 5 20
Insulin 1μM - + - + - + - +
Leptin 10nM - - + + - - + +

Because hyperleptinaemia and hyperinsulinaemia often occur simultaneously, we decided to assess the effect of a leptin-insulin combination on SREBP maturation. We found that at 5mM glucose, the combination of insulin and leptin increased level of both SREBP-1 mature form and precursor form. The same treatment at 20mM glucose significantly reduced levels of the mature and precursor form of SREBP-1 in comparison to levels at 5mM after the same treatment. The reduction was more striking with the mature form. While there were around 2-fold differences between levels of the precursor form, there was approximately a 5-fold difference between levels of the mature forms. Changes in level of SREBP mimicked changes in HSL expression in these cells very closely.

3.3.4 Changes in level of PPAR

The HSL promoter also contains binding sites for PPARs, which are a family of lipid-activated nuclear receptors. PPARgamma is another hormone and glucose responsive factor. whose level is induced by insulin in adipocytes (Krempler, Breban et al. 2000) and by glucose in HK-2 cells (Panchapakesan, Pollock et al. 2004).

The gamma isoform of this protein plays a prominent role in the control of lipid metabolism in macrophages. It is involved in the upregulation of CD36 receptor by oxLDL(Feng, Han et al. 2000). Component of the oxLDL particle such as 9-hydroxyoctadecadienoic acid and 13 hydroxyoctadecadienoic acid as well as other PPAR gamma ligands such as 15-deoxy-12,14 prostaglandin-J2 and thiazolidinedione increase CD 36 expression by activating PPAR gamma(Feng, Han et al. 2000). The PPAR gamma ligand TZD increases apolipoprotein AI-dependent efflux of cholesterol from both murine and human macrophages. Ligand activation of PPAR gamma leads to primary induction of oxysterol receptor LxR alpha, a direct transcriptional target of PPAR gamma-RxR heterodimers, followed by LxR-RxR activation of ABCA promoter(Chinetti, Lestavel et al. 2001).

Because of its prominent role in lipid metabolism, we decided to assess changes in its level in J774.2 macrophages. J774.2 cells were treated with high or low glucose for the designated time. Cells were lysed with RIPA buffer and equal amounts of protein loaded on to the gel. As shown in Figure 3.7, the level of PPAR gamma was not affected by insulin or leptin but PPAR gamma level was significantly reduced by high glucose.

3.3.5 Changes in levels of other transcription factors

We assessed changes in level of other transcription factors that could be involved in regulating HSL and which could potentially contribute to the effects of glucose described

above. As shown in Figure 3.8, we found that there were no changes in levels of c-Jun or CREB.

3.3.6 SREBP expression activates HSL promoter

Having found that SREBP maturation changes in parallel with changes in HSL expression, we proceeded to determine if overexpression of SREBP-1 affects activity of HSL promoter. An expression plasmid for SREBP-1 was co-expressed with pGL3 basic plasmid containing the -2682 to + 301 fragment of HSL promoter. The use of pGL3 basic instead of pGL3 enhancer plasmid in this transfection would reduce cross reactivity between SREBP-1 and the enhancer element. Transfection was carried out in CHO cells. Semi-confluent CHO cells were grown overnight in antibiotic-free media. Semi-confluent cells were transfected with lipofectamine and allowed to proliferate in antibiotic-free media containing 10% serum for 24 hours. Cells were lysed in luciferase assay buffer and readings taken by luminometer. As shown in Figure 3.9, we found that there was no change to readings from empty control plasmid following SREBP-1 co transfection. Using the plasmid containing the HSL promoter, SREBP-1 co transfection increased HSL promoter activity more than 2.5-fold.

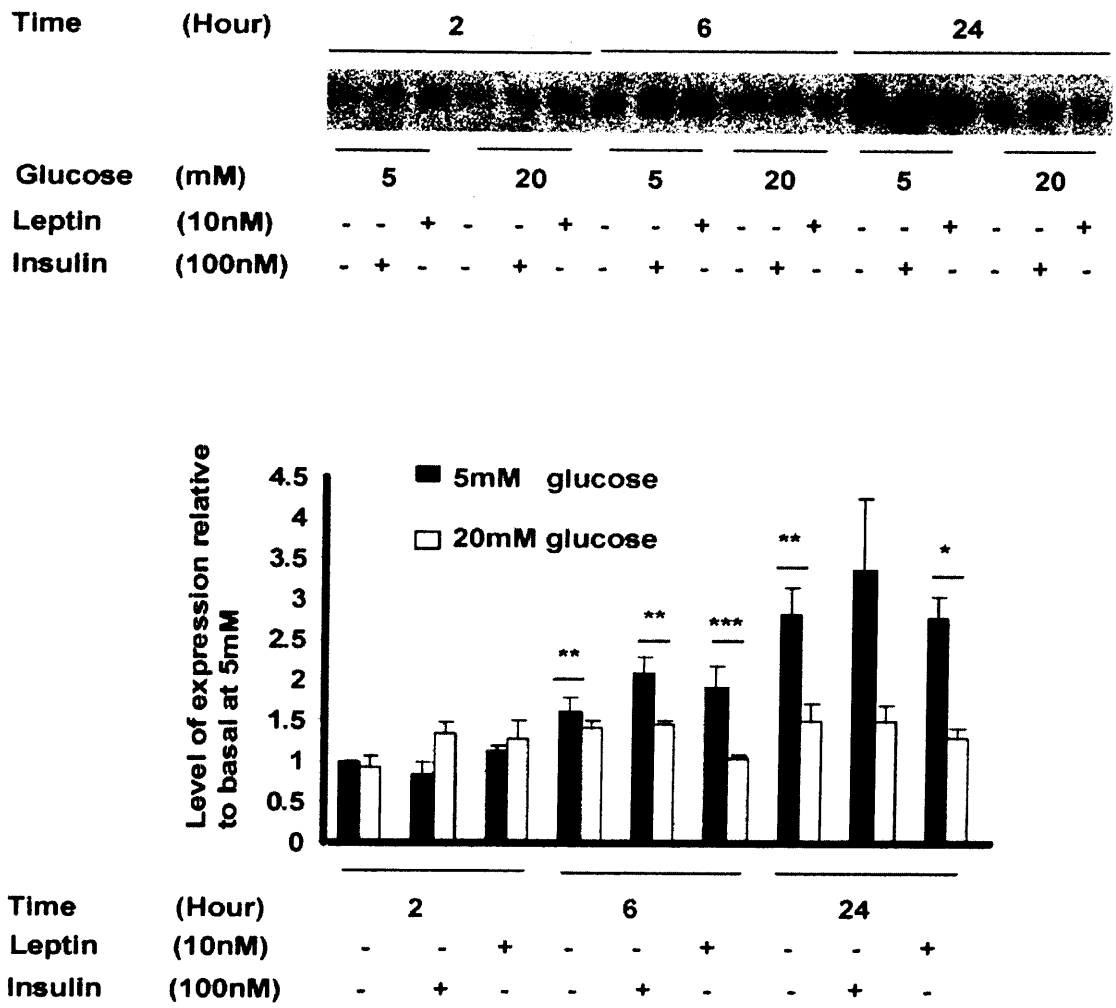


Figure 3.7 PPAR gamma level decreases when J774.2 cells were treated with glucose

J774.2 cells were incubated in serum free RPMI 1640 media containing 5mM or 20mM glucose supplemented with 2% serum overnight before further treatment in similar media for 2, 6 or 24 hours as indicated. Cells were lysed with RIPA buffer. Equal amount of protein was separated by SDS-PAGE electrophoresis and PPAR gamma detected by western blot. Blots were visualised by immunofluorescence. Relative level of PPAR gamma was quantified with Fuji images software. Data represent the mean ± SEM of three independent experiments. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicates differences where $P \leq 0.01$,** indicates $P \leq 0.05$ and *** indicates where $P \leq 0.09$.

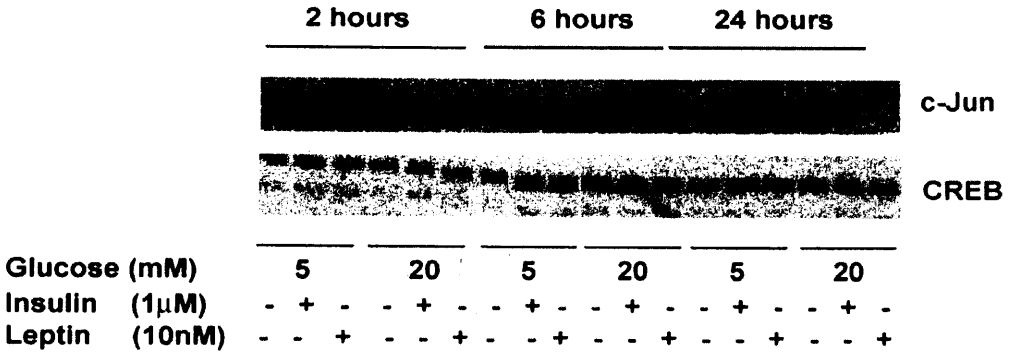


Figure 3.8 No change in level of CREB and c-Jun

J774.2 cells were incubated in serum free RPMI 1640 media containing 5mM or 20mM glucose supplemented with 2% serum overnight before further treatment in similar media for 2, 6 or 24 hours as indicated. Cells were lysed with RIPA buffer. Equal amount of protein was separated by SDS-PAGE electrophoresis and CREB or c-Jun gamma detected by western blot. Blots were visualised by immunofluorescence.

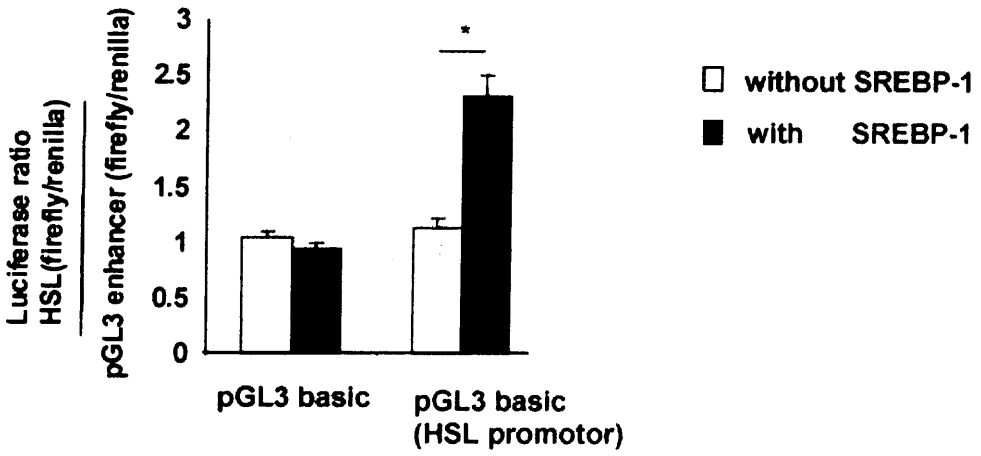


Figure 3.9 SREBP-1 co transfection increases HSL promotor activity

CHO cells were split at a 1 in 4 ratio in antibiotic-free media 24 hours before the start of transfection. Transfection was carried out on roughly 50% confluent cells. Cells were transfected with pGL3 basic plasmid or pGL3 basic plasmid containing -2682 to + 301 residue or HSL promoter with or without pCMV SREBP-1 plasmids. Renilla pTK plasmid was used as internal control. Transfection reagent used was lipofectamine. Amount of DNA was kept constant by addition of empty plasmid. After 24 hours, cell were lysed with luciferase assay buffer and readings taken by luminometer. The ratio between firefly and renilla was quantified. Experiments were performed three times in triplicate. Data represent the mean ± SEM of a representative triplicate. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicates differences where $P \leq 0.01$ and ** indicates $P \leq 0.05$.

3.4. Discussion

3.4.1 Glucose and PPAR gamma

Current evidence points to atheroprotective role of PPAR gamma. PPAR gamma agonists inhibit foam cell formation within the peritoneal cavities of hypercholesterolemic LDLR-/- mice (Li and C.K. 2000; Collins and G. 2001; Li, Binder et al. 2004) and in ApoE -/- mice fed high fat diet (Chen, Tamura et al. 2001.). Bone marrow transplanted from PPAR gamma null mice aggravates lesion development in LDL receptor-null mice. In humans, reduced carotid intimal thickening has been observed in diabetic patients treated with TZD, a specific activator of PPAR gamma (Minamikawa, Tanaka et al. 1998; Koshiyama, Shimono et al. 2001). In this experiment, we found that chronic incubation in high glucose significantly reduced PPAR gamma levels in J774.2 macrophages. This reduction in level would contribute to lesion development because the cells lose atheroprotective effects of PPAR gamma.

Mechanistically, reduction in level of PPAR gamma would cause reduced expression of ABCA, a cholesterol exporter and reduced cholesterol efflux from macrophages (Akiyama 2002). Lower levels of ABCA have been observed in leukocytes treated with high glucose. Perhaps glucose-induced reduction in PPAR gamma level is the underlying cause of the reduction (Albrecht, Simon-Vermot et al. 2004). Recently Osbourne et al showed that PPAR overexpression increased HSL promoter activity. Reduction in PPAR gamma in hyperglycaemia may contribute to lower HSL level observed in our experiments. However, it does not seem to be the only underlying reason for changes in level of HSL because there was no effect by insulin or leptin on PPAR gamma level at both high and low glucose.

3.4.2 Changes in SREBP level in J774.2 macrophages

SREBP was another candidate for our study because HSL promoter contains several SRE sites. We found that changes in level of SREBP-1 mimicked changes in HSL expression very closely although there was no change in SREBP-2. In contrast to findings in liver and fat cells where leptin decreases and insulin increases SREBP-1 mRNA level(Kakuma, Lee et al. 2002), we found that at 5mM glucose, there was no effect on SREBP-1 mature form in response to insulin, but leptin increased level of the SREBP mature form. Maturation of SREBP is controlled at the level of ER to golgi transport. SREBP forms a complex with SCAP protein in the ER (Sakai, Duncan et al. 1996). The complex is retained within the ER by interaction between SCAP and INSIG protein (Yang, Espenshade et al. 2002). Both sterol concentration and hormone affects this interaction but they exert their effects via two

separate mechanisms. In a sterol-rich environment, the interaction between INSIG and SCAP is enhanced through cholesterol-mediated conformational change of cytoplasmic loop of SCAP (Brown, Sun et al. 2002). This leads to ER retention by inhibiting Sar1-dependent binding of the COPII proteins Sec 23/24 to SCAP, inhibiting access of SCAP-SREBP complex to COPII coated vesicle in transitional ER (Espenshade, Li et al. 2002). Hormones, on the other hand, affect expression of INSIG protein. Insulin regulates SREBP-1 processing (Hegarty, Bobard et al. 2005) in part by decreasing the mRNA of INSIG-2 (Yabe, Komuro et al. 2003; Yellaturu, Deng et al. 2005). Downregulation of INSIG protein allows translocation of SREBP-SCAP to golgi complex for processing. Insulin stimulates processing of SREBP-1 through a PI3 kinase-mediated pathway (Hegarty, Bobard et al. 2005).The requirement for PI3-kinase in the maturation of SREBP explains the effect of insulin and leptin on SREBP-1 maturation in J774.2 cells. O'Rourke et al found that in J774.2 cells, leptin is a more potent activator of PI3-kinase than insulin (O'Rourke, Yeaman et al. 2001). There is a high probability that administration of leptin would activate the PI3-kinase-dependent maturation process of SREBP-1. We have not identified the exact process this entails, but leptin might downregulate INSIG-2 mRNA in a similar manner to insulin.

We found that levels of SREBP-1 mature form in leptin-treated cells were lower in high glucose in comparison to the level at 5mM glucose. There was a significant reduction with leptin treatment in comparison with the level at 20mM glucose alone. Our result agrees with previous finding in muscle where leptin reduces the effects of high glucose on SREBP-1 levels in skeletal muscle after refeeding (Commerford, Peng et al. 2004). The reasons for this are not entirely clear but it is possible to speculate. Chronic flux through the hexosamine pathway may be responsible for downregulation of leptin-stimulated PI3-kinase activation. Previous studies have shown that increased glycosylation of intracellular proteins reduces the sensitivity of postreceptor insulin signalling events. Reduced IRS-1/IRS-2 signalling (Patti 1999) as well as reduced PKB/Akt activity (Vosseller, Wells et al. 2002) has been observed with increasing glycosylation. Although PI3 kinase can be activated by hexosamine flux, resulting downstream cascade pathways activation by hexosamine flux differs from downstream pathways activated by insulin. For example, PI3-kinase activation from hexosamine does not increase GLUT4 translocation. Activation of PI3 kinase by hexosamine may even dampen PI3 kinase response to further hormone treatment (Filippis, Filippis et al. 2002). We may speculate that dampening of PI3-kinase response to leptin treatment and glycosylation of signalling proteins were responsible for reduced SREBP-1 processing when cells were pre-treated with high glucose.

Although insulin did not affect maturation of SREBP-1, we found that it potentiated the effects of leptin. Together, addition of insulin and leptin increased both precursor form and mature form of SREBP-1. This suggests that a combination of leptin and insulin affects the expression of SREBP-1, possibly at the transcriptional level. When cells were pre-treated with 20mM glucose before combined treatment of insulin and leptin, there was significant reduction in level of SREBP precursor form together with an even greater reduction in the level of SREBP mature form. The level of the precursor form was half of that at 5mM glucose with similar treatment while the level of mature form decreased 5 fold. The observation suggests that there is a reduction in expression as well as a reduction in maturation in 20mM glucose.

Additive effects between leptin and insulin have previously been reported. At the whole body level, leptin appears to act as an insulin-sensitising factor in rat. Leptin markedly enhanced insulin action on both inhibition of hepatic glucose production, stimulation of glucose uptake (Barzilai, Wang et al. 1997) and glucose utilisation(Sivitz, Walsh et al. 1997).Animals previously treated with leptin show an increase in glucose uptake and glycogen synthesis in peripheral tissues in response to insulinaemic clamp as compared with food restricted litter mate (Barzilai, She et al. 1999). Leptin increases glucose uptake and decarboxylation and glycogen synthesis in incubated soleus muscle (Ceddia, William et al. 1999) and glycogen synthesis in hepatocytes (Cohen, Werrmann et al. 1998). In adipocytes, chronic exposure to leptin for 15 hours increased basal and insulin-induced glucose decarboxylation (Ceddia, William et al. 1998) as well as Krebs cycle activity (Ceddia, William et al. 2000).

Inhibitor studies and overexpression studies in 3T3-L1 cells (Nadeau, Leitner et al. 2004) and primary cultures of rat hepatocytes (Matsumoto, Ogawa et al. 2002) have shown that PI3-kinase and PKB (Fleischmann and Iynedjian 2000) mediate the effects of insulin on SREBP-1 mRNA expression in addition to the aforementioned effects on SREBP-1 processing. In J774.2 cells incubated in low glucose, additive effects between insulin and leptin would enhance PI3 kinase activation. This would lead to accelerated SREBP-1 processing and the generation of increasing amount of mature SREBP-1. In the nucleus, accumulation of mature SREBP-1 would enhance the activation of SREBP-1 promoter, as the proximal SREBP-1 promoter contains SRE which allows SREBP to activate its own promoter (Amemiya-Kudo 2000) causing further increases in SREBP-1 expression. In high glucose, insulin may potentiate o-linked glycosylation of proteins along PI3-kinase signalling cascades. This would inhibit leptin signalling and SREBP-1 mRNA expression and protein processing. Alternatively, accumulation of cholesterol in these cells may

contribute to inhibition of processing. Accumulation of cholesterol ester and increased uptake of free cholesterol in high glucose, hyperinsulinaemia and hyperleptinaemia (O'Rourke 2002) may increase intracellular cholesterol content beyond a threshold where SREBP-1 processing is inhibited completely by ER retention. The SREBP promoter also contains NF-Y and Sp-1 sites which contribute to regulation of SREBP promoter by insulin and leptin. Transactivation by SREBP-1 in other gene promoters such as that of FAS and ATP-dependent citrate lyase requires simultaneous binding of Sp1 or NF-Y to adjacent sites (Magana 2000; Moon 2000). Rapid and enhanced expression of SP1 by insulin has been documented in rat liver and in rat hepatoma cells (Pan, Solomon et al. 2001). Similar phenomena may exist in J774.2 macrophages. Interestingly, comparatively lower levels of SREBP-1 have been observed in muscle of diabetic rat (Guillet-Deniau, Mieulet et al. 2002) as well as in adipose tissue and muscle of diabetic human subjects (Sewter, Berger et al. 2002). This reduction may contribute to the general dyslipidaemia observed in the diabetic state. Correction of the processing could be another viable strategy for diabetic treatment.

We found that high glucose on its own significantly increased SREBP-1 mature form. Our result agrees with a previous report in contracting myotubes where glucose upregulates both precursor and mature forms of SREBP-1 (Guillet-Deniau, Pichard et al. 2004). The reduction in PPAR gamma in response to chronic glucose could contribute to glucose-induced increases in the mature form of SREBP in J774.2 macrophages. PPAR gamma response element has been found on INSIG promoter and rosiglitazone induces expression of INSIG protein. Down regulation of PPAR gamma in high glucose would decrease level of INSIG protein, allowing more processing of SREBP-1 (Kast-Woelbern, Dana et al. 2004).

Furthermore PPAR is an inhibitor of SREBP -1 expression. It reduces binding of LxR/RxR ligand to the LxR response element on SREBP promoter by competing for RxR (Yoshikawa, Ide et al. 2003). Downregulation of PPAR gamma would increase availability of free RxR, allowing more LxR/RxR complex formation. LxR/RxR, a receptor for oxysterol, is a potent activator of SREBP-1 expression (Yoshikawa, Shimano et al. 2001). This may seem paradoxical since oxysterol is an inhibitor of SREBP processing. However, oxysterol is not a very potent inhibitor of SREBP-1 processing and activation of SREBP-1 expression by oxysterol may help maintain level of SREBP-1 even under sterol-suppressant condition. This will ensure production of fatty acid to provide acyl residues for cholesterol esterification process even under high cholesterol. Together the process would protect cells from free cholesterol-induced cytotoxicity. However, the contribution of LxR to SREBP-1 expression in macrophages is uncertain. Synthetic LxR ligands do not activate SREBP-1 expression in J774.2 cells to the same extent as in adipocytes although activation of ABCA was

comparable (Quinet, Savio et al. 2004). This discrepancy may be caused by higher expression of LxR beta in relation to LxR alpha in J774.2 cells (Quinet, Savio et al. 2004). Since the SREBP-1 promoter is more responsive to LxR alpha (Peet, Turley et al. 1998; Alberti, Schuster et al. 2001), significantly lower level of LxR alpha in these cells might not permit it to act as an activator of SREBP-1 expression.

3.4.3 SREBP activation of HSL promoter

Our expression analysis with the luciferase reporter gene subsequently showed that an increase in SREBP-1 expression led to increased activity of the HSL promoter construct. However, we have not analysed the exact mechanism. SREBP might bind to and directly activate the promoter. An almost identical response pattern between changes in level of SREBP mature form and HSL expression as well as the presence of several SRE sites on HSL promoter suggests that this could be the case. On the other hand, overexpression of SREBP-1 is known to activate the PPAR gamma promoter (Fajas, Schoonjans et al. 1999). SREBP overexpressed in our system could activate the expression of PPAR gamma, which then activates HSL promoter. It would be necessary to perform band shift analysis to assess this query. Furthermore, SREBP is a weak activator of transcription. It always acts in concert with other co-activators such as CBP (Gaus, Kritharides et al. 2004). We have not analysed the contribution of other factors in the process. It is uncertain whether SREBP is a sufficient activator, or a necessary but insufficient contributor to the activation. RNAi studies should shed more light in the overall process. Thus far we have done our analysis in a mouse system, it would be necessary to perform similar analysis in a human system before a meaningful conclusion could be reached.

3.4.4 Therapeutic consideration

Because SREBP is involved in the regulation of lipid synthesis in many metabolically important tissues such as muscle, fat and liver, it has been attracting much attention as a potential new target for lipid lowering drug. A new class of compound that directly binds to the sterol regulatory element binding protein cleavage activating protein (SCAP) has been manufactured (Grand-Perret, Bouillot et al. 2001). The compound binds directly to SCAP, enhancing the maturation of SREBP. In hamster, administration of SCAP ligand increases LDLr expression in the liver. This decreases plasma cholesterol in both very low density lipoprotein and low density lipoprotein fraction without affecting level of high density lipoprotein. In addition to its benefit on circulating lipid profile, the finding in this chapter suggests that this compound may correct the decrease in HSL level occurring as a result of hyperglycaemia and hormonal changes. By increasing SREBP maturation, this compound

might enhance HSL expression and activity, leading to a reduction in cholesterol ester accumulation.

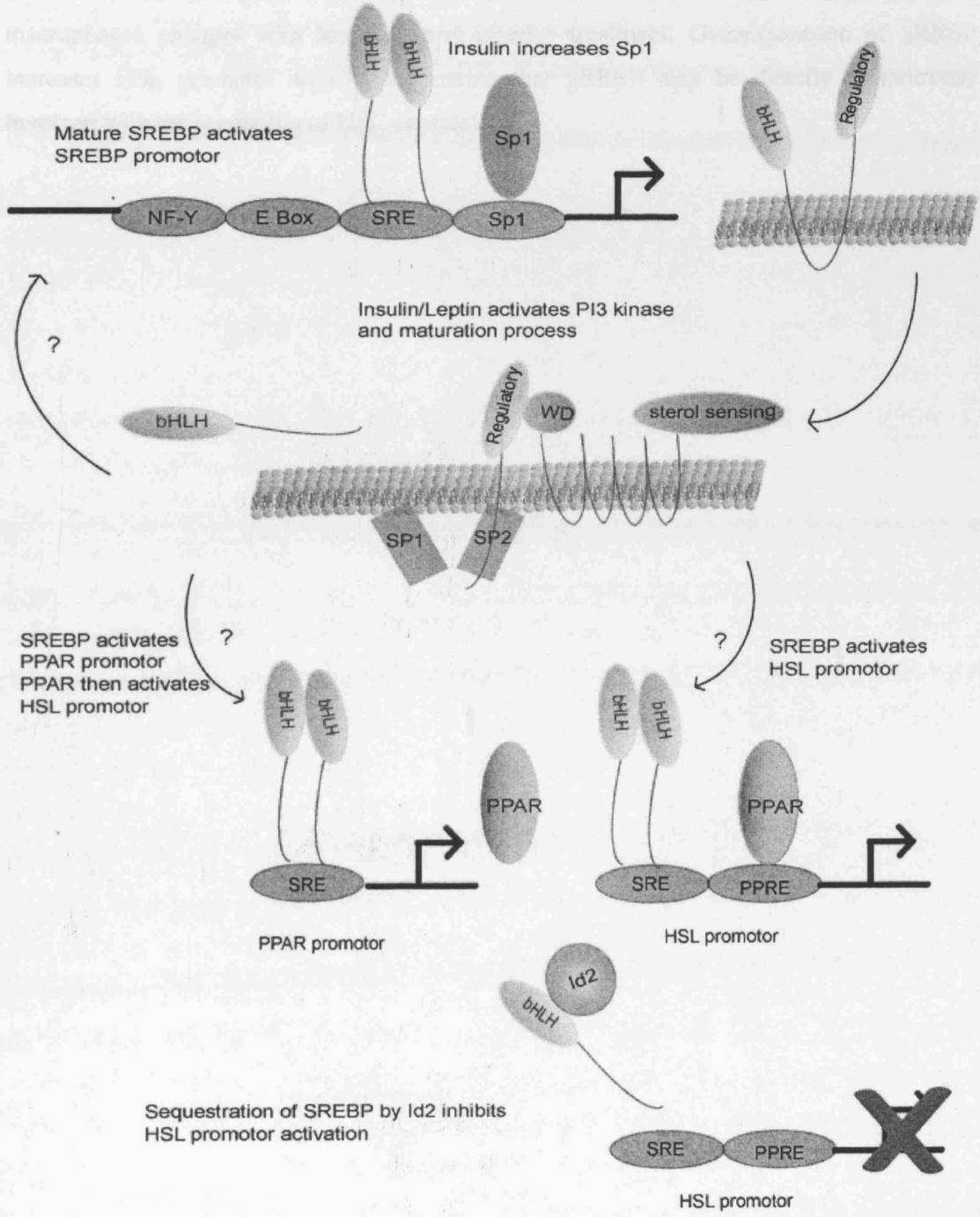


Figure 3.10 Summary of mechanism linking SREBP-1 to changes in HSL promoter activity. Insulin increases the amount of mature SREBP-1 by increasing the expression of SREBP-1 precursor and activating the cleavage process. Mature SREBP-1 may activate HSL promotor directly through SRE sites. Alternatively, SREBP-1 may increase the expression of PPAR gamma. PPAR gamma then activates HSL through PPRE sites.

3.5 Conclusion

In conclusion, this chapter provides evidence that level of SREBP mature form in macrophages changes with hormone and glucose treatment. Overexpression of SREBP increases HSL promoter activity, suggesting that SREBP may be directly or indirectly involved with the regulation of HSL expression.

Chapter 4 Results

Glucose regulates levels of the transcriptional repressor Id2 and these effects are mediated by the hexosamine pathway

4.1 Abstract

Hyperglycaemia has been well documented as an accelerator of atherosclerotic plaque development. High levels of glucose have many proatherogenic effects on macrophages, which are the most abundant cells in fatty streak lesions. Some of these effects are mediated through its impact on macrophage intracellular lipid metabolism. For example, it facilitates cholesterol ester deposits by increasing ACAT activity and reduces HSL activity in response to insulin and leptin. This results in increased lipid uptake and reduced cholesterol efflux. Microarray analysis was utilised in an attempt to assess changes in macrophage gene expression in response to hyperglycaemia. The study identified Id2, a negative regulator of an important regulator of lipid metabolism such as SREBP, as a gene responsive to chronic hyperglycaemia in J774.2 macrophages. The mechanisms linking hyperglycaemia to increases in Id2 level have not been elucidated, therefore this study attempted to analyse this. We found that glucose upregulated Id2 protein level independently of osmolarity, protein kinase C or glucose-6-phosphate. On the other hand, changes in Id2 level occurred in response to glucosamine and overexpression of GFAT. Upregulation of Id2 required glutamine and was inhibited by azaserine, an inhibitor of GFAT. We conclude that hexosamine flux is required for the glucose induced changes in Id2 level. Id2 itself was modified by o-linked glycosylation in response to glucosamine addition. Hence changes in hexosamine flux may affect both expression of Id2, its rate of degradation and other aspects of its functions. Finally, we considered functional implications of Id2 accumulation. We found that co-transfection of Id2 attenuated SREBP-1-mediated activation of the HSL promoter. Our observation suggests that accumulation of Id2 under chronic hyperglycaemia may have adverse effects on intracellular lipid homeostasis.

4.2 Introduction

The formation of atherosclerotic lesions starts early in life and fatty streaks have even been found in human foetal aorta (Napoli, D'Armiento et al. 1997). These fatty streaks then increase rapidly in prevalence and extent during the 15-34 age span (Strong, Malcom et al. 1999). Fatty streaks may not have clinical symptoms but they are the precursor of more advanced plaques which contribute to cardiovascular diseases. Prevalence of cardiovascular disease is much higher in the diabetic population and it contributes to premature mortality in diabetes (Kannel 1979; Stamler 1993; Stout 1993; Lee 2000; Khaw 2001; Bonora 2003; Hegazi 2003; Lee 2003; Otel 2003; McGuire 2004). This increase in cardiovascular diseases can be accounted for by accelerated carotid atherosclerosis development (Wagenknecht 2003).

The fact that the risk of cardiovascular disease is reduced in patients with good glycaemic control suggests that hyperglycaemia is one of the cardiovascular risk factors associated with diabetes (UKPDS 1998; Esposito 2004). Hyperglycaemia has many proatherogenic effects on macrophages, which are one of the main cell types found in the atherosclerotic plaque lesion. High glucose increases monocyte-endothelial cell attachment (Ikeda U 1998) and transmigration (Rattan 1996) as a result of increased production of chemoattractant molecules such as MCP-1(Piemonti 2003) and increased generation of oxidised LDL (Kawamura, Heinecke et al. 1994). Hyperglycaemia increases the uptake of modified lipoprotein by inducing the expression of scavenger receptor type A in human monocyte-derived macrophages through PKC and NAD(P)H dependent pathway(Fukuhara-Takaki K 2004). Hyperglycaemia also enhances the expression of leptin-like oxidized LDL receptor-1 (LOX-1), a newly discovered receptor for oxidized LDL, in macrophages (Li 2004).

Once taken up, lipoprotein cholesterol is metabolised by major pathways of cholesterol homeostasis in macrophages that ensures a suitable balance of free cholesterol and cholesterol ester accumulation. Lysosomal acid lipase catalyses lysosomal hydrolysis of cholesterol ester and triglyceride from lipoproteins. This process releases free fatty acids, mono- and di-triglyceride. Excess free fatty acid is re-esterified and stored in the cytosol as cholesterol ester by ACAT-1. Macrophages cannot catabolise cholesterol. However, they can release cholesterol to extracellular receptors, which remove cholesterol from peripheral cells to the liver, after cholesterol ester is hydrolysed by neutral cholesterol ester hydrolase. Hyperglycaemia affects the balance of this cycle. O' Rourke et al showed that priming of macrophages with high glucose increased esterification activity of ACAT and reduced cholesterol ester hydrolysis by HSL, leading to increased cholesterol ester deposition instead of free cholesterol production in response to insulin and leptin (O'Rourke 2002). In

combination with a reduction in ABCA-1 level (Albrecht, Simon-Vermot et al. 2004), hyperglycaemia reduces free cholesterol efflux from macrophages.

It is vital to uncover previously unrecognised glucose-responsive genes in macrophages which may contribute to changes in level of hormone-sensitive lipase protein, intracellular lipid imbalance and lesion development. To identify glucose-responsive genes, a DNA microarray study was performed using RNA prepared from J774.2 murine macrophage cells treated with high glucose (Gronning, L unpublished result). One of the genes identified was Id2, an inhibitor of differentiation. As far as atherosclerosis development is concerned, Id2 is a very interesting candidate because of its ability to bind to and inhibit SREBP-1 (Moldes 1999). SREBP is a regulator of lipid homeostasis, which suggests changes in Id2 could have a wider involvement in lipid regulation. Both Id1 and Id3 are known to change in response changes in lipid (Busch, Cordery et al. 2002) and glucose level (Wice, Bernal-Mizrachi et al. 2001) in β-cells, further suggesting that Id proteins might play vital role in cellular response to changes in nutrient status. Upregulation of Id2 mRNA in muscle, liver and adipose tissue of ob/ob mice (Vicent 1998) as well as high resistance of Id2 knockout mice to diet-induced atherosclerosis (Aoki 2003) also imply that Id2 plays a role in metabolic disease progression.

A number of pathways have been identified which can mediate the effects of glucose on gene expression and protein levels in the cell. These include osmotic stress, direct effects of glycolytic intermediates, protein kinase C and the hexosamine pathway. Changes in osmotic stress exerted by increasing glucose concentration can affect gene expression through PKC beta (Assert, Scherk et al. 2001; Massucco, Mattiello et al. 2005) or stress-induced BMK1(Suzaki, Yoshizumi et al. 2004). Internalised glucose, which has been metabolised to glucose-6-phosphate, has been shown to affect expression of lipogenic enzymes in adipocytes and the liver (Foufelle 1998). High glucose also induces *de novo* DAG generation through inhibition of glycolytic enzyme GADPH (Du 2003). This leads to the build-up of the glycolytic intermediate dihydroxyacetone phosphate, which can be used for DAG synthesis after reduction to glycerol-3-phosphate. Activation of PKC by glucose is well documented in macrophages (Hill, Kwon et al. 1998; Li 2004). Alternatively, the hexosamine biosynthesis pathway has been postulated to be the intracellular glucose sensing mechanism and the downstream substrate of this pathway such as glucosamine affects the expression of many genes. Because knowledge of the signalling cascade can lead to targets for therapeutic intervention in the future, work in this chapter will attempt to elucidate the signalling pathways involved in regulating Id2 expression.

4.3 Result

4.3.1 Microarray analysis identifies Id2 as a glucose responsive gene in macrophages

Gene array analysis was performed by Dr Line Gronning, Oslo. Briefly, RNA was isolated from J774.2 macrophages that had been treated with 5mM or 20mM glucose for 6 hours under serum-free conditions. Isolated RNA was reverse transcribed using Superscript II reverse transcriptase and labelled with Cy3 and Cy5 dye to form first strand cDNA. These cDNA probes were hybridised with cDNA elements from the NIA mouse 15K cAMP array preparation. Fluorescent images of hybridised microarrays were captured using GSI Lumonics 4000 scanner and ScanArray software. Results from the analysis using GeneSpring software identified several genes affected by raising glucose concentration as shown in Figure 4.1. Id2 mRNA level demonstrated significant changes of 1.7fold induction with p=0.05 statistical significance.

Gene	Genbank	Fold
Id2	BG073126	Up 1.7 x , p=0.05
Galpha-14	BG084187	Up 2.0 x , p=0.04
MacMARCKS	BG077510	Up 1.9 x , p=0.02
Calreticulin	BG064608	Down 0.5 , p=0.01

Figure 4.1 Selected glucose upregulated genes in macrophages as assayed by Microarray analysis.

4.3.2 J774.2 macrophages express Id2 mRNA

Microarray technology still has many limitations. Noise, systematic error and random error from each step of preparation compound to distort final reading. More importantly, because of the lack of microarray-probe sequence information, there is no guarantee that an oligomer or cDNA probe actually represents the gene that it is supposed to (van Bakel and Holstege 2004). To confirm that Id2 mRNA were expressed in J774.2 cells, total RNA from J774.2 cells were extracted and reverse transcribed. Resulting cDNA was amplified by PCR using primer specific for Id2 and separated on the gel. The result qualitatively showed that Id2 mRNA were present in J774.2 macrophages (Figure 4.2). Having confirmed that Id2 was present in J774.2 cells, we proceeded with the next stages of our analysis.

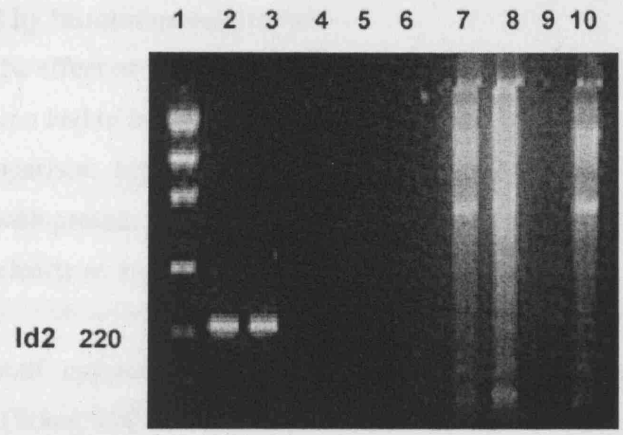


Figure 4.2 : mRNA of Id2 is expressed in J774.2 murine macrophages J774.2 macrophages were serum-starved overnight before being treated with high glucose. RNA was extracted from J774.2 macrophages by trizol reagent. Subsequently, mRNA was reversed transcribed by Superscript II enzyme. The product was amplified by PCR using Taq DNA polymerase. Primers for PCR were designed using Primer 3 program and sequence NM_010496 from NCBI. The forward primer was 5' CTC CAA GCT CAA GGA ACT GG 3' and the reverse primer was 5' ATT CAG ATG CCT GCA AGG AC 3'. The product was separated on the gel as followed.

Lane 1 : 1kb ladder

Lane 2 : RT-PCR product of mRNA from J774.2 macrophages treated with high glucose

Lane 3 : RT-PCR product of mRNA from J774.2 macrophages treated with high glucose

Lane 4 : Superscript II without mRNA

Lane 5 : RNA without Superscript II

Lane 6 : Taq polymerase without RT product

Lane 7 : RT product

Lane 8 : RT product

Lane 9 : PCR without RT product

Lane 10 : RT product without Taq polymerase

4.3.3 Detection of Id2 by immunoprecipitation

We decided to verify the effect of glucose on Id2 at the protein level. In order to clearly detect Id2, immunoprecipitation had to be performed from cell lysates containing an equal amount of total protein. A comparison between the control with protein G alone and complete immunoprecipitation with protein G bead and primary antibody against Id2 demonstrated that Id2 could be detected clearly as an Mr 15kDa band (Figure 4.3A).

Id2 was found in both cytosolic and nuclear fraction of cell lysates, depending on differentiation stages (Wang 2001; Liu, Ding et al. 2002). In order to optimise extraction protocol, we compared detection of Id2 after extraction using nuclear fraction preparation protocol or extraction using Sucrose buffer from samples with equal amount of total protein. As shown in Figure 4.3B, significantly larger amount of Id2 could be immunoprecipitated from Sucrose buffer-based lysate. This observation suggested that a substantial amount of Id2 in J774.2 cells resided in the cytosol; hence further experiments were performed with Sucrose buffer.

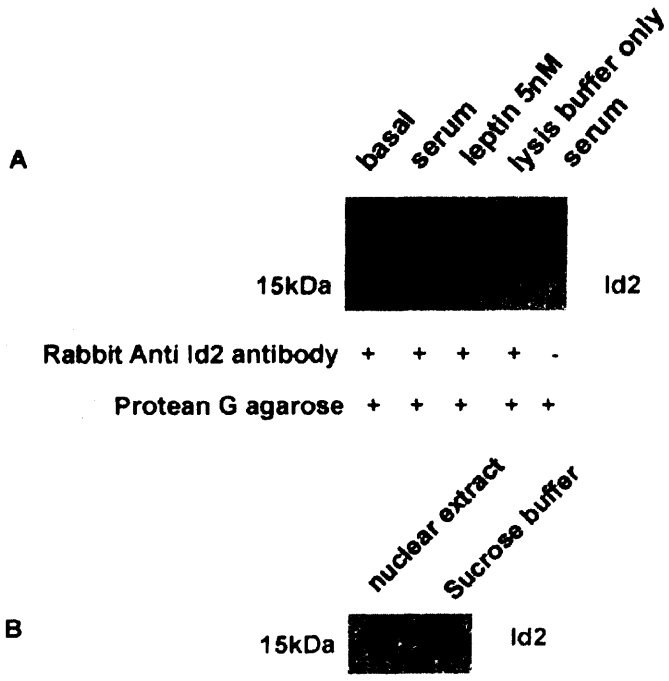


Figure 4.3 Optimisation of Id2 immunoprecipitation Figure 4.3A J774.2 cells were starved overnight prior to treatment with serum or leptin for 4 hours and extracted with Sucrose buffer. Protein concentration was measured with BCA kit. Equal amounts of protein from each sample were used in immunoprecipitation. Extracts were incubated overnight with or without anti-Id2 antibody before being incubated for 1 hours with 30ul of protein G bead. Beads were washed twice in lysis buffer before the addition of sample buffer and separation on agarose gel. Blot were probed with anti Id2 antibody. Figure 4.3B J774.2 cells were starved overnight prior to treatment with serum. Cells were extracted with either RIPA buffer or nuclear extraction protocol. Protein concentrations were normalised before nuclear extract preparation and immunoprecipitations were performed on normalised samples.

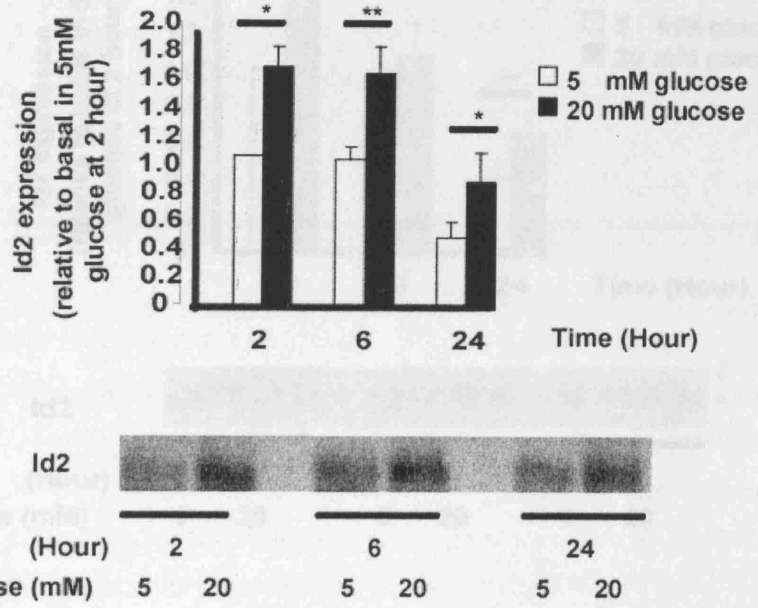
4.3.4 Glucose increases Id2 protein levels

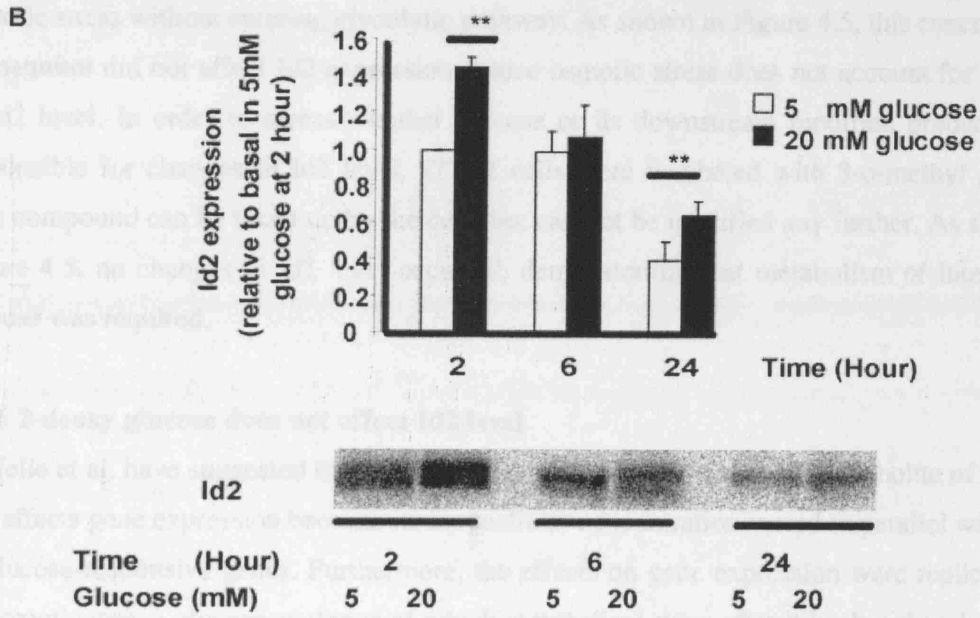
The western blot technique was employed to assess changes in protein level of Id2. In a previous study by O'Rourke et al., changes in expression of HSL occurred after cells were pre-treated in high glucose overnight, before being treated for further 2, 6, or 24 hours (O'Rourke 2002). In order to assess whether glucose influenced Id2 level after such extended time points, J774.2 cells were pre-treated overnight at 5mM or 20mM glucose before further treatment for 2, 6 or 24 hours. To assess changes in Id2 level in a conventional time course, J774.2 cells were serum-starved overnight under 5mM glucose (Figure 4.4B) or 0.5 mM glucose (Figure4.4C) before being treated with the designated glucose concentration. Upregulation of Id2 by glucose could be detected in both cases. The upregulation occurred significantly after 2 hours incubation and was maintained up to 24 hours time point.

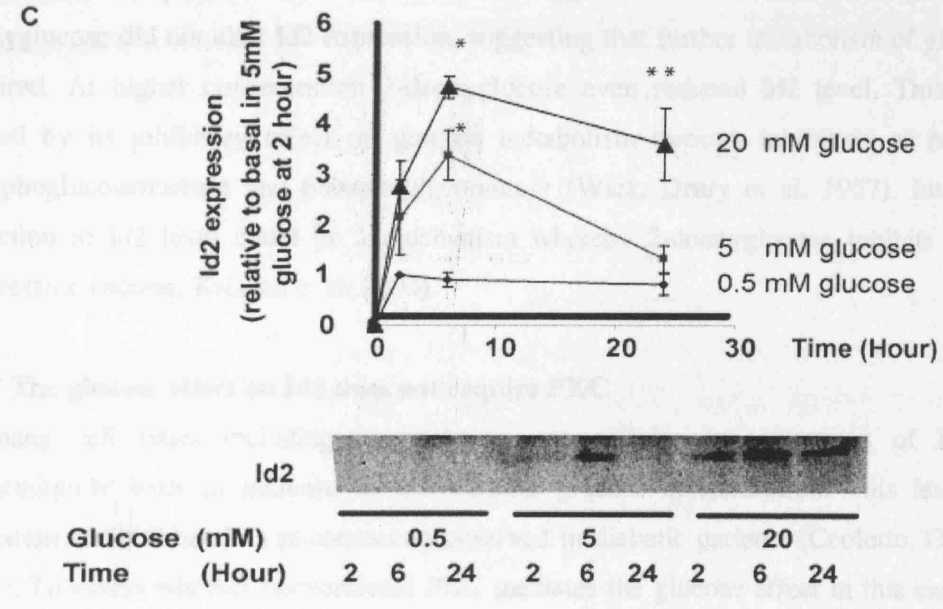
Figure 4.4 Glucose increases the Id2 protein level in J774.2 macrophages

(A) J774.2 macrophages were incubated with serum-free RPMI 1640 media containing either 5mM or 20mM glucose and supplemented with 0.2% fatty acid free BSA overnight before further incubation of 2, 6 or 24 hours as indicated. (B) J774.2 macrophages were incubated with RPMI 1640 containing 5mM glucose and supplemented with 0.2% BSA overnight before cells were treated for further 2, 3 or 24 hours with 5mM or 20mM glucose. (C) J774.2 macrophages were incubated with RPMI 1640 media containing 0.5 mM glucose and supplemented with 0.2% BSA for 16 hour. The medium was then replaced with fresh medium containing 0.5 mM , 5mM or 20mM glucose for 2, 6 or 24 hours. After the designated incubation Id2 was immunoprecipitated and total Id2 protein level protein was determined by Western blotting. Relative level of Id2 was quantified. Data represent the mean ± SEM of three independent experiments. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicates differences where $P \leq 0.01$ and ** indicates $P \leq 0.05$.

A


Id2 expression
(relative to basal in 5mM
glucose at 2 hour)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
*
5 mM glucose
20 mM glucose
2
6
24
Time (Hour)
Id2
Time (Hour) 2 6 24
Glucose (mM) 5 20 5 20 5 20


B
Id2 expression (relative to basal in 5mM glucose at 2 hour)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
5 mM glucose
20 mM glucose
2
6
24
Time (Hour)
Id2
Time (Hour) 2 6 24
Glucose (mM) 5 20 5 20 5 20


C
Id2 expression (relative to basal in 5mM glucose at 2 hour)
6
5
4
3
2
1
0
*
*
**
20 mM glucose
5 mM glucose
0.5 mM glucose
0
10
20
30
Time (Hour)
Id2
Glucose (mM) 0.5 5 20
Time (Hour) 2 6 24 2 6 24 2 6 24

4.3.5 Osmotic stress does not mediate the effect of glucose on Id2 level and the glucose effect requires modification of internalised glucose

Changes in osmolarity caused by increased extracellular glucose concentration may potentially affect gene expression. In order to assess whether changes in Id2 occurred because of osmotic stress, J774.2 cells were incubated with 20 mM mannitol to mimic the potential osmotic effects of high glucose. Because mannitol is not taken up by the cells, it exerts osmotic stress without entering glycolytic pathway. As shown in Figure 4.5, this concentration of mannitol did not affect Id2 expression; hence osmotic stress does not account for changes in Id2 level. In order to assess whether glucose or its downstream modified products were responsible for changes in Id2 level, J774.2 cells were incubated with 3-o-methyl glucose. This compound can be taken up by the cells but can not be modified any further. As shown in Figure 4.5, no changes in Id2 level occurred, demonstrating that metabolism of internalised glucose was required.

4.3.6 2-deoxy glucose does not affect Id2 level

Foufelle et al. have suggested that glucose-6-phosphate is the signalling metabolite of glucose that affects gene expression because its intracellular concentration varied in parallel with level of glucose-responsive genes. Furthermore, the effects on gene expression were replicated by 2-deoxyglucose, a glucose analogue of which metabolism stops after it is phosphorylated into 2-deoxyglucose-6-phosphate, which accumulates in the cells (Foufelle, Girard et al. 1998). In order to analyse whether glucose-6-phosphate was the signalling metabolite, J774.2 were treated with varying concentration of 2-deoxyglucose. As shown in Figure 4.6, 2-deoxyglucose did not alter Id2 expression, suggesting that further metabolism of glucose was required. At higher concentration 2-deoxyglucose even reduced Id2 level. This could be caused by its inhibitory effect on glucose metabolism through inhibition of hexokinase, phosphoglucoisomerase and phosphoglucomutase (Wick, Drury et al. 1957). Interestingly, reduction in Id2 level could be a mechanism whereby 2-deoxyglucose inhibits cell cycle progression (Maher, Krishan et al. 2004).

4.3.7 The glucose effect on Id2 does not require PKC

In many cell types including macrophages, intracellular concentrations of DAG rise concomitantly with an increase in extracellular glucose concentration. This leads to the activation of PKC-beta2, a phenomenon observed in diabetic patients (Ceolotto, Gallo et al. 1999). To assess whether conventional PKC mediates the glucose effect in this case, J774.2

cells were treated with PMA for 16 hours to downregulate alpha and beta isoforms of PKC. Subsequently, cells were treated with high or low glucose. As shown in Figure 4.7A, PMA downregulated both conventional isoforms of PKC, however this did not affect induction of Id2 expression by high glucose as shown in Figure 4.7B. The result ruled out involvement of PKC alpha and beta.

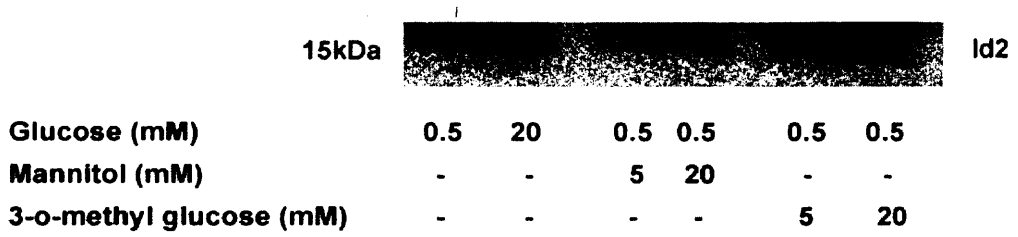


Figure 4.5 Osmotic stress and unmodified glucose does not affect Id2 level

J774.2 macrophages were incubated with serum free RPMI 1640 media (supplemented with 0.2% BSA) containing 0.5mM or 20mM and the indicated amount of Mannitol. After 16 hours the medium was replaced with fresh RPMI 1640 media (supplemented with 0.2% BSA) containing 0.5mM or 20mM and the indicated amount of Mannitol. After 6 hours Id2 was immunoprecipitated from the lysate and total Id2 protein levels were determined by Western blotting. A representative blot from three independent experiments is shown.

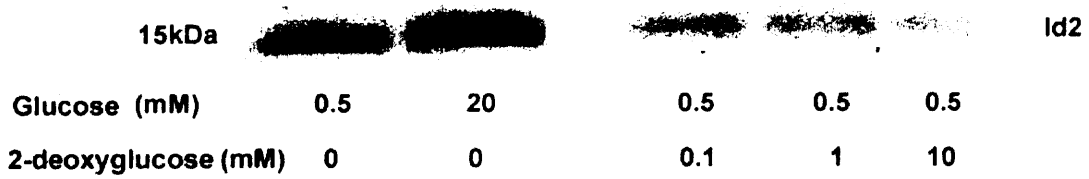


Figure 4.6 2-deoxyglucose does not affect Id2 level

J774.2 macrophages were incubated with serum free RPMI 1640 media containing 0.5 or 20 mM glucose supplemented with 0.2% BSA. After 16 hours the medium was replaced with fresh RPMI 1640 media (supplemented with 0.2% BSA) containing 0.5mM glucose or 20mM glucose with or without the indicated amount of 2-deoxy glucose . After 6 hours Id2 was immunoprecipitated and total Id2 protein levels was determined by Western blotting. A representative blot from three independent experiments is shown.

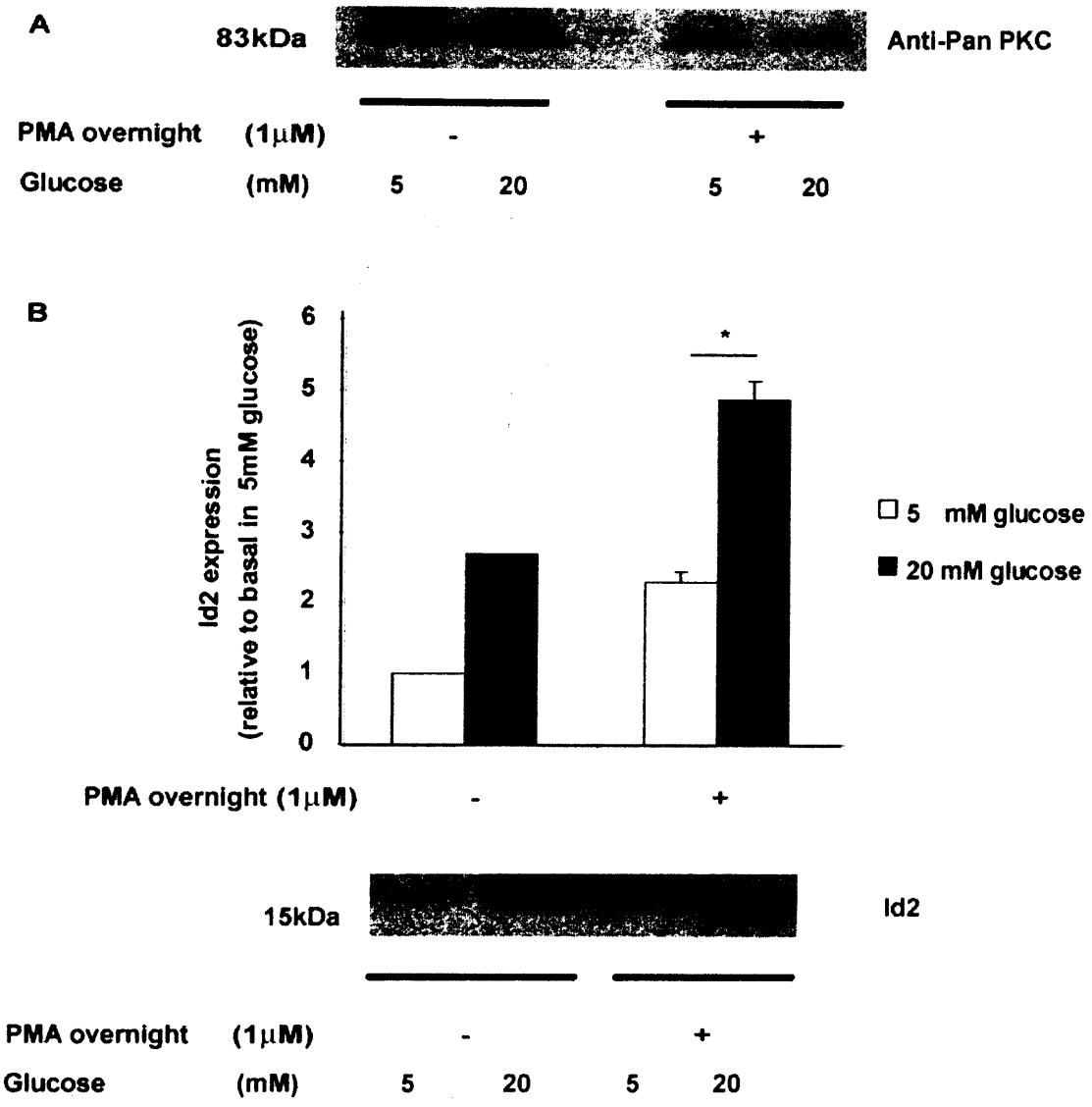


Figure 4.7 The effect of glucose on Id2 expression does not require the typical form of PKC (PKC alpha or beta)

J774.2 macrophages were incubated with serum-free RPMI 1640 media (supplemented with 0.2% BSA and 5mM or 20mM glucose) with or without 1μM PMA overnight. In the morning the medium was replaced with fresh serum-free RPMI 1640 media containing either 5mM or 20mM glucose with or without 1μM PMA. After 4 hours cell lysate was prepared and Id2 immunoprecipitated. Protein level of PKC alpha and beta (A) and Id2 protein levels (B) were determined by Western blotting. A representative blot from three independent experiments is shown.

4.3.8 Hexosamine flux mediates the effect of glucose on Id2 expression

Another pathway postulated to mediate the intracellular effect of glucose is the hexosamine biosynthesis pathway (Mc Clain 2002). Around 2-3% of glucose derived from glucose-6-phosphate is transformed into glucosamine-6-phosphate through GFAT-catalysed reaction (Marshall, Bacote et al. 1991). Glucosamine-6-phosphate is the precursor of UDP-N Acetyl glucosamine which can be added to proteins by O-linkage through serine or threonine residues. O-linked glycosylation often alters the functions of transcription factors and signalling cascade components, ultimately causing changes in pattern of gene expression. In order to assess whether this pathway is involved with Id2 expression, J774.2 cells were treated with glucosamine. Glucosamine can be taken up by the GLUT family of glucose transporter. It is phosphorylated intracellularly to form glucosamine-6-phosphate, an end product of a GFAT-catalysed reaction.

As shown in Figure 4.8A, We found that low concentrations of glucosamine (0.2 or 2 mM) mimicked the effect of 20mM glucose in increasing Id2 protein level. The GFAT-catalysed reaction requires glutamine, hence we assessed whether glutamine withdrawal would alter the effect of glucose on Id2 expression. As shown in Figure 4.8D, removal of glutamine inhibited high glucose induced Id2 expression.

To further confirm that hexosamine flux is involved, we overexpressed GFAT in J774.2 cells using adenoviral transfection. The transfection was kindly carried out by Dr. Gnudi (King's College London). The cACCMVpLpA-GFAT vector was prepared by cloning GFAT cDNA into multiple cloning site of vector pACCMVpLpA. The recombinant adenovirus expressing GFAT cDNA was generated by co-transfection of the plasmids cACCMVpLpA-GFAT and pJM17 in HEK293 cells using calcium phosphate transfection kit. J774.2 macrophages were co-transfected with β-galactosidase control or GFAT adenovirus at 100 multiplicity of infection (plaque forming unit per J774.2 macrophages) for 4 hours. Virus was removed and cells were incubated in media containing 5mM glucose and serum overnight. In the morning cells were incubated with fresh media containing 5mM glucose and serum before being lysed (Burt, Gruden et al. 2003). Id2 expression clearly increased in the transfected samples in comparison with nontransfected ones as shown in Figure 4.8C.

Finally, to confirm the involvement of this pathway we inhibited GFAT using azaserine, a specific inhibitor of the enzyme. As shown in Figure 4.8B, glucose effect was abolished following azaserine administration. Together, we conclude from the evidence that hexosamine flux is involved with the glucose effect on Id2 protein level.

4.3.9 Glucose effect on Id2 level requires PI 3 kinase

We investigated whether known cell signalling pathways might mediate the effects of the hexosamine pathway as there is previous evidence to indicate that both the PI 3-kinase and ERK/MAP Kinase pathways can play a role in mediating the effects of high glucose. In rat adipose tissues, the effects mediated via the hexosamine pathway were found to require the activation of PI3-kinase (Filippis, Filippis et al. 2002). Alternatively, high glucose activates Erk in vascular smooth muscle cells (Kawamura, Yokote et al. 2004). To test the involvement of PI3 kinase and Erk in our system, J774.2 cells were treated with wortmannin LY294002 or PD98059 prior to stimulation with high glucose. As shown in Figure 4.9, wortmannin and LY294002 pre-treatment inhibited the glucose effect in J774.2 cells at the concentration that specifically inhibits PI3 kinase..

According to Fillipis et al, activation of PI3-kinase by hexosamine flux leads to the activation of atypical and novel isoforms of PKC through the production of phosphoinositides. Although we have ruled out activation of conventional isoforms, these atypical and novel isoforms could be involved in J774.2 cells. We have not studied this possibility in detail, neither has the mechanism linking hexosamine flux to PI3-kinase activation been elucidated. It is possible that the reduction in the glucose effect caused by PI-3-kinase inhibitors could be due to a reduction in glucose uptake and hence in hexosamine flux. Wortmannin is known to inhibit basal glucose uptake in Sertoli cells (Riera 2003) and in rat skeletal muscle (Acitores 2005). Application of wortmannin to J774.2 cells will most likely reduce uptake of glucose in to the cells. This would reduce glucose flux through hexosamine pathway, thereby inhibiting the glucose effect on Id2 expression.

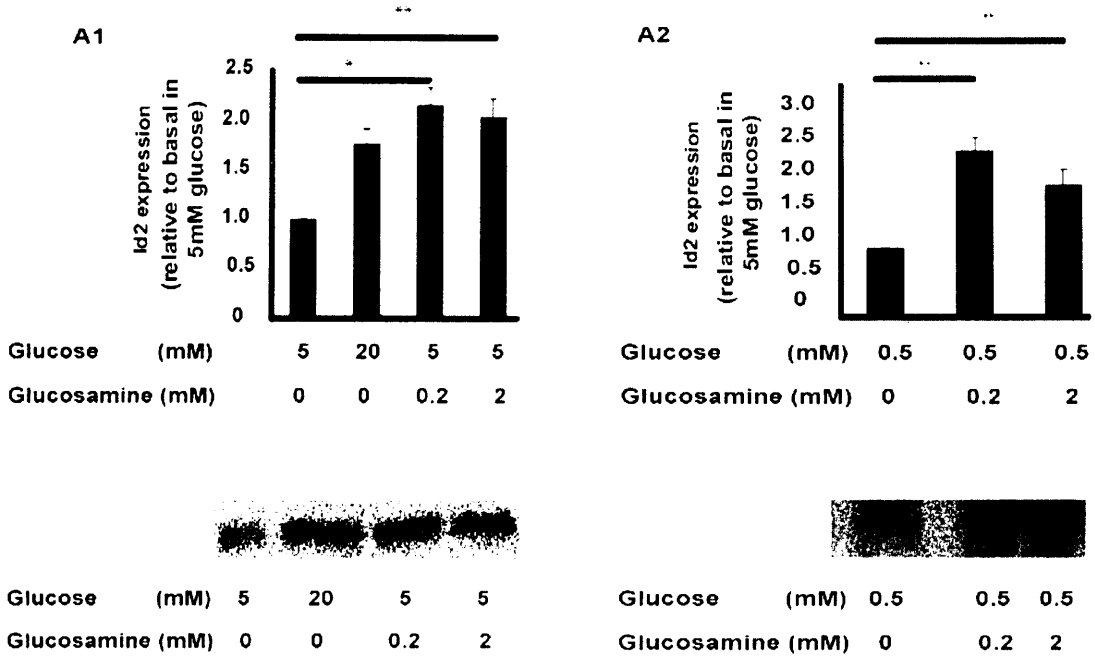


Figure 4.8 (A) Glucosamine mimics the glucose effect on Id2 protein level in J774.2 macrophages

A.1: J774.2 macrophages were incubated with serum-free RPMI 1640 media (supplemented with 0.2% fatty acid free BSA) containing either 5mM or 20mM glucose or the indicated amount of glucosamine and supplemented with 0.2% BSA for 16 hour. The medium was then replaced with fresh medium containing 5 mM or 20mM glucose and the indicated amount of glucosamine. After 6 hours Id2 was immunoprecipitated. Id2 protein level was determined by Western blotting. Quantitation of several experiments was performed. Data represent the mean ± SEM of three independent experiments performed in duplicate. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicate differences where $P \leq 0.05$ and ** indicates where $P \leq 0.01$.

A.2: J774.2 macrophages were incubated with RPMI 1640 media containing 0.5 mM or 20mM glucose or the indicated amount of glucosamine and supplemented with 0.2% BSA for 16 hour. The medium was then replaced with fresh medium containing 0.5mM or 20mM glucose and the indicated amount of glucosamine. After 6 hours Id2 was immunoprecipitated and Id2 protein level was determined by Western blotting. Quantitation of several experiments was performed. Data represent the mean ± SE of four independent determinations performed in duplicate. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where ** indicate differences where $P \leq 0.05$.

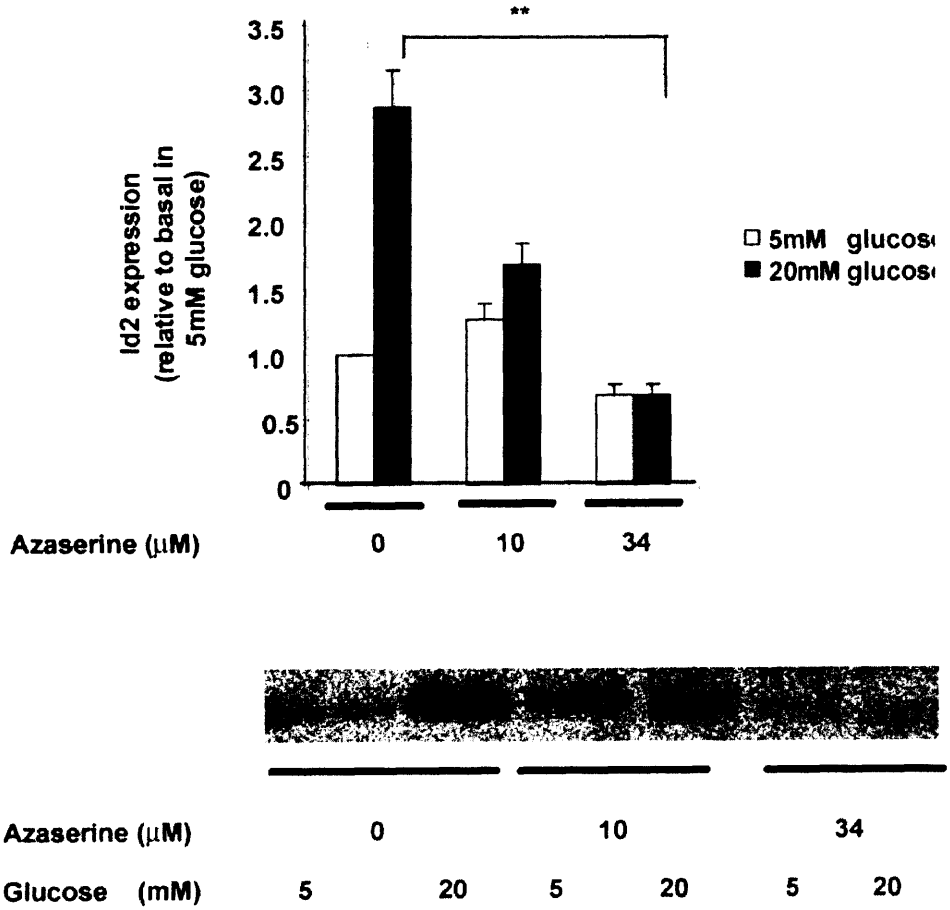


Figure 4.8 (B) Azaserine represses the increase in Id2 level with glucose in a dose dependent manner

J774.2 macrophages were incubated with serum-free RPMI 1640 media (supplemented with 0.2% BSA) with or without the indicated amount of Azaserine. After 45 minutes 15mM glucose was added to designated plates to obtain the final concentration of 20mM. The cells were incubated for further 16 hour before the medium was replaced with fresh RPMI1640 media containing either 5mM or 20mM glucose with or without the indicated amount of Azaserine. After 6 hours cell lysate was prepared and Id2 immunoprecipitated. Id2 protein levels were determined by Western blotting. Quantitation of several experiments was performed. Data represent the mean ± SEM of three independent determinations performed in duplicate. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where ** indicates differences where $P \leq 0.05$.

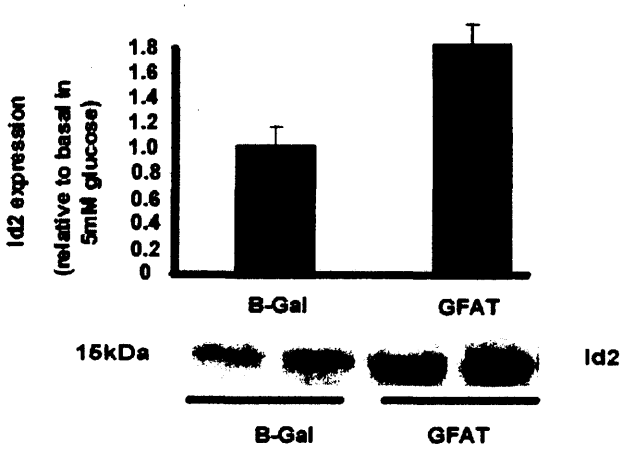


Figure 4.8 (C) GFAT overexpression increases Id2 expression in J774.2 cells

GFAT were overexpressed in J774.2 cells using adenoviral transfection. After 24 hours cells were lysed and Id2 immunoprecipitated. Id2 levels were determined by western blotting. Data represent the mean of a duplicate experiment.

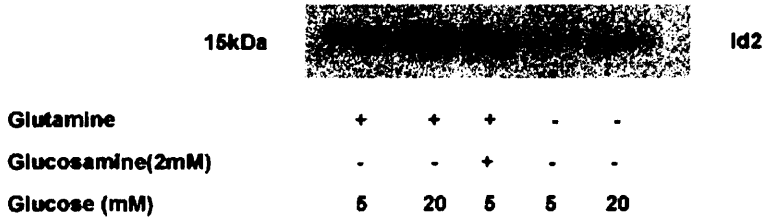


Figure 4.8 (D) The effect of glucose on Id2 protein level requires glutamine

J774.2 macrophages were incubated for 16 hour with serum free RPMI 1640 media containing 5mM or 20mM glucose with or without glutamine. The medium was replaced with fresh RPMI 1640 media (supplemented with 0.2% BSA) containing 5mM or 20mM glucose with or without glutamine. After 6 hours cell lysate was prepared and Id2 immunoprecipitated . Id2 protein levels were determined by Western blotting. A representative blot from three experiments is shown.

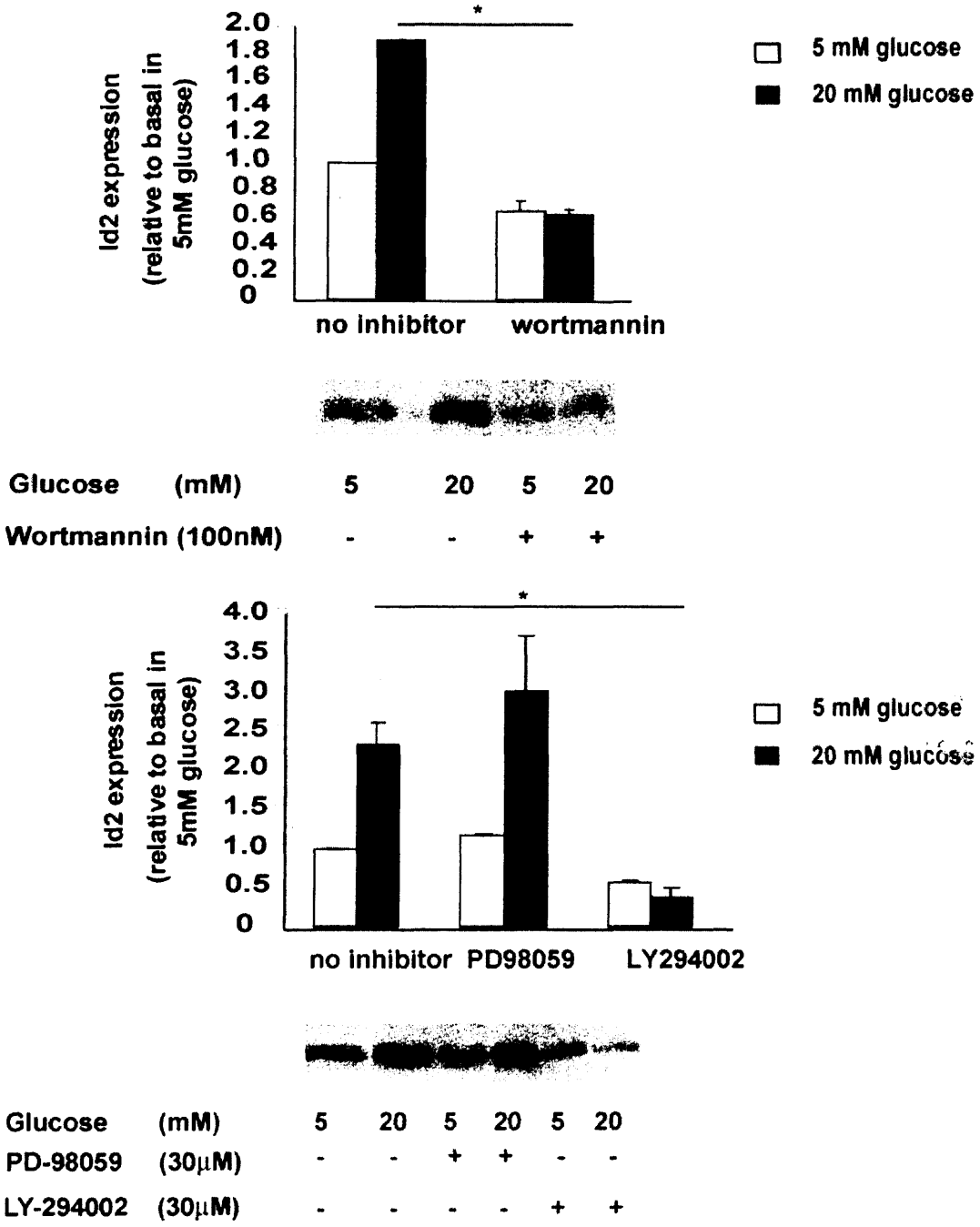


Figure 4.9 Glucose effect requires PI3 Kinase

J774.2 macrophages were incubated with 100nM wortmannin or 30μM LY 294002 or 30μM PD 98059 for 45 minutes before being treated with 20mM glucose or 5mM glucose for 16 hours. Id2 was immunoprecipitated from the lysate and relative level determined by western blotting. Result is a representative of three separate experiments. Significant differences are indicated where * indicates differences where P≤0.01.

4.3.10 Id2 itself is modified by O-linked glycosylation

The other pathway that might mediate the effects of hexosamines on Id2 levels could be through direct o-linked glycosylation of protein via o-glycosyl transferase (OGT). O-linked glycosylation usually occurs on the same residue as GSK3 phosphorylation sites (Kamemura, Hayes et al. 2002). Our analysis of the amino acid sequence of Id2 revealed that Id2 might be a target of GSK 3 phosphorylation. Its sequence (SDHSLGISRSKTPVDD) contains three successive serine/threonine residues in the correct SxxxS consensus. As shown in Figure 4.10, protein sequence alignment using ClustalW software (Thompson, Higgins et al. 1994) illustrates that these sites are conserved in mouse, humans, rat, rainbow trout and zebra fish. The GSK3 phosphorylation consensus is specific to Id2. It has not been found on Id1, Id3 or Id4. Consequently we carried out a preliminary experiment to assess whether O-linked glycosylation could be detected on immunoprecipitated Id2. According to existing literature, o-linked glycosylation increases with glucosamine and insulin administration. J774.2 cells were treated with glucosamine or insulin under serum free condition or with 10% serum. Cells were lysed with glycosylation-specific buffer supplemented with O-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc). PUGNAc, is a GlcNAc analogue that specifically inhibits peptide O-GlcNAc-β-N-acetylglucosaminidase (O-GlcNAcase) *in vivo* (Haltiwanger, Grove et al. 1998) and *in vitro* (Dong and Hart 1994) without affecting other components of the O-linked glycosylation machinery (Haltiwanger, Grove et al. 1998). Id2 was immunoprecipitated from the cell lysate and the western blot probed with CTD110.6 antibody (Comer, Vosseller et al. 2001). The antibody was raised in mouse against synthetic glycopeptide YSPTS(O-GlcNAc)PSK. ELISA analysis has shown that the antibody reacts with both O-GlcNAc-serine and O-GlcNAc-threonine residues from synthetic proteins as well as from proteins modified *in vivo* (Comer, Vosseller et al. 2001).

As shown in Figure 4.11A a 15kDa band could be detected by CTD110.6 antibody when cells were treated with glucosamine and insulin. Under serum-free condition shown in Figure 4.11B, 15kDa band could be detected with glucosamine alone, glucosamine in combination with insulin and in high glucose. Membranes were stripped and reprobed with total Id2 antibody and relative glycosylation analysed. As shown in the graphical representation, the highest proportion of glycosylation occurs when cells were treated with 20mM glucose together with 2mM glucosamine. Compared to the ratio at 5mM, the proportion of glycosylation increased with 20mM glucose, 2mM glucosamine and 2mM glucosamine with insulin. If the total amount of Id2 is taken into account, there will be the highest amount of glycosylated Id2 intracellularly when cells were treated with 2mM glucosamine together with 100nM insulin.

Figure 4.10 Protein sequence alignment by ClustalW

```
CLUSTAL W (1.82) multiple sequence alignment Id2
mouse            MKAFSPVRSVRKNS----LSDHSLGISRSKTPVDDPMSLLYNMNDCYSKLKELVPSIPQN 56
hamster          MKAFSPVRSVRKNS----LSDHGLGISRSKTPVDDPMSLLYNMNDCYSKLKELVPSIPQN 56
rat              MKAFSPVRSVRKNS----LSDHSLGISRSKTPVDDPMSLLYNMNDCYSKLKELVPSIPQN 56
human            MKAFSPVRSVRKNS----LSDHSLGISRSKTPVDDPMSLLYNMNDCYSKLKELVPSIPQN 56
chicken          MKAFSPVRSVRKNG----LSEHNLGISRSKTPVDDPMSLLYNMNDCYSKLKELVPSIPQN 56
Xenopus          MKAFSPVRSVRKSS----LTEHSLGIARSKTPVDDPMSLLYNMNDCYSKLKELVPSIPQN 56
trout            MKAISPVRSFRKNSSNLS--EHSLGISRSKTPVDDPLSLLYNMNDCYSKLKELVPSIPQN 58
fish_zebra_      MKAISPVRSFRKSSASVTTTEHSLGISRSKTPVDDPLSLLYNMNDCYSKLKELVPSIPQN 60
                 ***:*****.**..      :*.***:*********:**********************

mouse            KKVTKMEILQHVIDYILDLQIALDSHPTIVSLHHQ-RPGQNQASRTPLTTLNTDISILSL 115
hamster          KKVTKMEILQHVIDYILDLQIALDSHPTIVSLHHQ-RPGQNQASRTPLTTLNTDISILSL 115
rat              KKVTKMEILQHVIDYILDLQIALDSHPTIVSLHHQ-RPGQNQTSRTPLTTLNTDISILSL 115
human            KKVSKMEILQHVIDYILDLQIALDSHPTIVSLHHQ-RPGQNQASRTPLTTLNTDISILSL 115
chicken          KKVSKMEILQHVIDYILDLQIALDSHPSIVSLHHQ-RPGQNAASRTPLTTLNTDISILSL 115
Xenopus          KKVSKMEILQHVIDYILDLQLTLDSHPSIVSLHHLPRVGGN-TSRTPLDPLNTDISILSL 115
trout            KNVSKMEILQHVIDYILDLQIALDSNVAITSHHHP-RPGQA-TPRTPLTTLNTDISILSL 116
fish_zebra_      KNVSKMEILQHVIDYILDLQIALDSNSAITS-LHP-RAGQG-TSRTPLTTLNTDISILSL 117
                 *:*:*****************::***: :*.*  *  * *   :.**** .**********

mouse            QASEFPSELMSNDSKVLCG 134
hamster          QASEFPSELMSNDSKVLCG 134
rat              QASEFPSELMSNDSKVLCG 134
human            QASEFPSELMSNDSKALCG 134
chicken          QAAEFPSELMASDSKALCG 134
Xenopus          QAAEFSSEFT-DESKSLCP 133
trout            QSPEFPSDLITDDSRTLHR 135
fish_zebra_      QTPEFPSDLITEDSRTLYR 136
                 *:.**.*::  .:*: *

CLUSTAL W (1.82) multiple sequence alignment
Id2human         MKAFSPVRSVRKNSLS---------------DHSLG----------ISRSK--------- 26
Id2mouse         MKAFSPVRSVRKNSLS---------------DHSLG----------ISRSK--------- 26
Id4human         MKAVSPVRPSGRKAPSGCGGGELALRCLAEHGHSLGGSAAAAAAAAAARCKA------AE 54
Id4mouse         MKAVSPVRPSGRKAPSGCGGGELALRCLAEHGHSLGGSAAAAAAAAAARCKA------AE 54
Id3human         MKALSPVRGCYEAVCCLS-------------ERSLA----------IARGRG------KG 31
Id3mouse         MKALSPVRGCYEAVCCLS-------------ERSLA----------IARGRG------KS 31
Id1human         MKVASGSTATAAAGPSCALKAGKTASGAGEVVRCLS-----EQSVAISRCAGGAGARLPA 55
Id1mouse         MKVASGS-AAAAAGPSCSLKAGRTA---GEVVLGLS-----EQSVAISRCAG---TRLPA 48
                 **. *          .                *.           :*

Id2human         TEVDDP-MSLLYNMNDCYSKLKELVPSIPQNKKVSKMEILQHVIDYILDLQIALDSHPTI 85
Id2mouse         TEVDDP-MSLLYNMNDCYSKLKELVPSIPQNKKVTKMEILQHVIDYILDLQIALDSHPTI 85
Id4human         AAADEPALCLQCDMNDCYSRLRRLVPTIPPNKKVSKVEILQHVIDYILDLQLALETHPAL 114
Id4mouse         AAADEPALCLQCDMNDCYSRLRRLVPTIPPNKKVSKVEILQHVIDYILDLQLALETHPAL 114
Id3human         PAAEEP-LSLLDDMNHCYSRLRELVPGVPRGTQLSQVEILQRVIDYILDLQVVLA----- 85
Id3mouse         PSTEEP-LSLLDDMNHCYSRLRELVPGVPRGTQLSQVEILQRVIDYILDLQVVLA----- 85
Id1human         LLDEQQVNVLLYDMNGCYSRLKELVPTLPQNRKVSKVEILQHVIDYIRDLQLELNSESEV 115
Id1mouse         LLDEQQVNVLLYDMNGCYSRLKELVPTLPQNRKVSKVEILQHVIDYIRDLQLELNSESEV 108
                    ::    *  :** ***:*:.*** :* . :::::*****:***** ***: * : .

Id2human         VS-LHHQRP-QNQASRTPLTTLNTDISILSLQASEFPSELMS-----------NDSKALC 132
Id2mouse         VS-LHHQRPGQNQASRTPLTTLNTDISILSLQASEFPSELMS-----------NDSKVLC 133
Id4human         LRQPPPPAPPHHPAGTCPAAPPRTPLTALNTDP---AGAVNK-----------QGDSILC 160
Id4mouse         LRQPPPPAPPLHPAGACPVAPPRTPLTALNTDP---AGAVNK-----------QGDSILC 160
Id3human         -----EPAP-------GPPDGPHLPIQTAELAP----ELVIS-----------NDKRSFC 118
Id3mouse         -----EPAP-------GPPDGPHLPIQTAELTP----ELVIS-----------KDKRSFC 118
Id1human         GTPGGRGLP-----VRAPLSTLNGEISALTAEAACVP----------------ADDRILC 154
Id1mouse         GTTGGRGLP-----VRAPLSTLNGEISALAAEVRSESEYYIILQWETEATGGGCPPSLLF 163
                        *    :   *    .  :          .                    :

Id2human         G---- 133
Id2mouse         G---- 134
Id4human         R---- 161
Id4mouse         R---- 161
Id3human         H---- 119
Id3mouse         H---- 119
Id1human         R---- 155
Id1mouse         RRIAI 168
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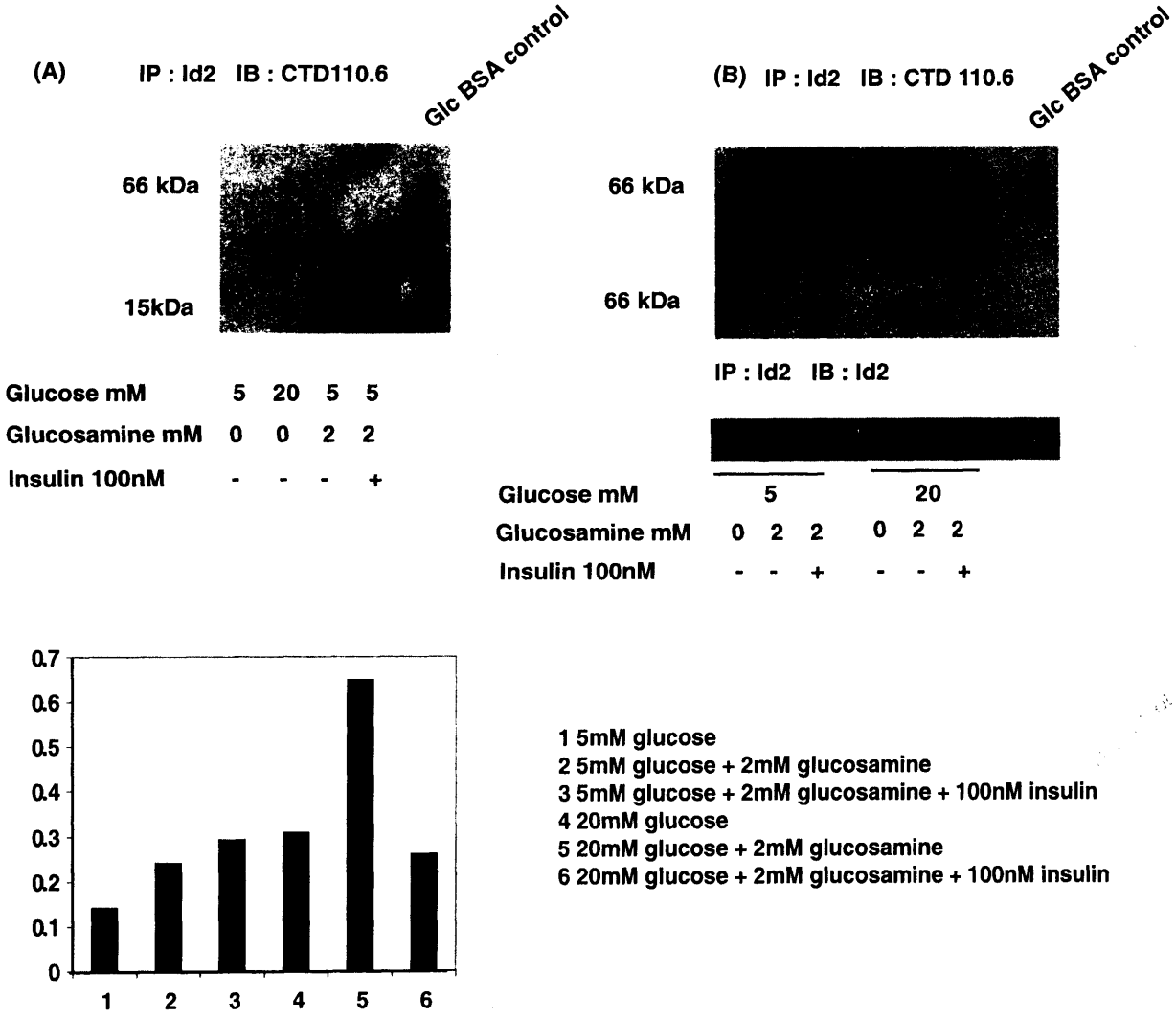


Figure 4.11 O'linked glycosylation of Id2

J774.2 cells were incubated with 5mM glucose or 20mM glucose in RPMI 1640 media supplemented with 10% serum (A) or with 0.2% BSA (B) for 24 hours. Cells were lysed with glycosylation-specific lysis buffer supplemented with 10μM PUGNAC inhibitor. Id2 was immunoprecipitated and separated by gel electropheresis. Membranes were blotted with CTD110.6 antibody specific for O-linked glycosylated residue and detected by chemiluminescence. Membrane from (B) was stripped and reprobed with anti-Id2 antibody. Representative blots from three separate experiments are shown. The graph (C) represents proportion of glycosylation in relation to total Id2 from (B).

This preliminary experiment suggests that Id2 could be modified by O-linked glycosylation . We observed less glycosylation under serum-supplemented condition. Several factors in the serum may activate phosphorylation of proteins. Because phosphorylation and o-linked glycosylation usually compete for the same residue on proteins, high level of phosphorylation under serum-supplemented condition would decrease o-linked glycosylation. Insulin may increase o-linked glycosylation by inhibiting GSK3 phosphorylation of Id2; this would allow o-linked glycosylation to occur on the site usually occupied by phosphorylation. Alternatively, insulin is known to promote glucose uptake in monocytes expressing GLUT1 and GLUT3 (Daneman 1992; Estrada 1994). An increase in glucose uptake in response to insulin in our cells could increase glucose flux through hexosamine pathway. This would increase Id2 levels.

4.3.11 Degradation of Id2 slows down under high glucose

Id2 is degraded via the ubiquitin-proteasome pathway (Fajerman, Schwartz et al. 2004). Because O-GlcNAc modification is an endogenous inhibitor of the proteasome (Zhang, Su et al. 2003), we postulated that degradation rate of Id2 might decrease under high glucose condition. To assess this hypothesis, J774.2 cells were incubated under high or low glucose for 6 hours before addition of 10ug/ml cycloheximide, an inhibitor of translation. Cells were harvested at designated time point. As shown in Figure 4.12, the degradation rate of Id2 slowed under high glucose condition. It is quite likely that the slower rate of degradation contributes to higher levels of Id2 detected under high glucose.

4.3.12 Testing the effect of glucose on Id2 levels in other cell types

Many genes in liver, fat and muscle are responsive to glucose and dysregulation of their expression in many cases is associated with metabolic syndrome. Id2 has been found in these tissues but its response to high glucose has not been analysed, therefore we carried out similar treatment with HepG2 cells, L6 myocytes, isolated cardiomyocytes, isolated soleus muscle and 3T3-L1 adipocytes. As shown in Figure 4.13, we found no change in Id2 in response to glucose in cardiomyocytes (Figure 4.13C) and L6 myocytes (Figure 4.13A). In soleus muscle, we found changes in response to 20 mM glucose only when cells were simultaneously treated with glutamine. Glucosamine also upregulated Id2 in these tissues (Figure 4.13B). In HepG2 cells, both 20mM glucose and 0.2mM glucosamine significantly upregulated Id2 expression. In differentiated 3T3-L1 cells incubated under 5mM glucose during differentiation, changes in level of Id2 in response to glucose was not statistically significant but changes in response to 0.2mM and 2mM glucosamine were significant.

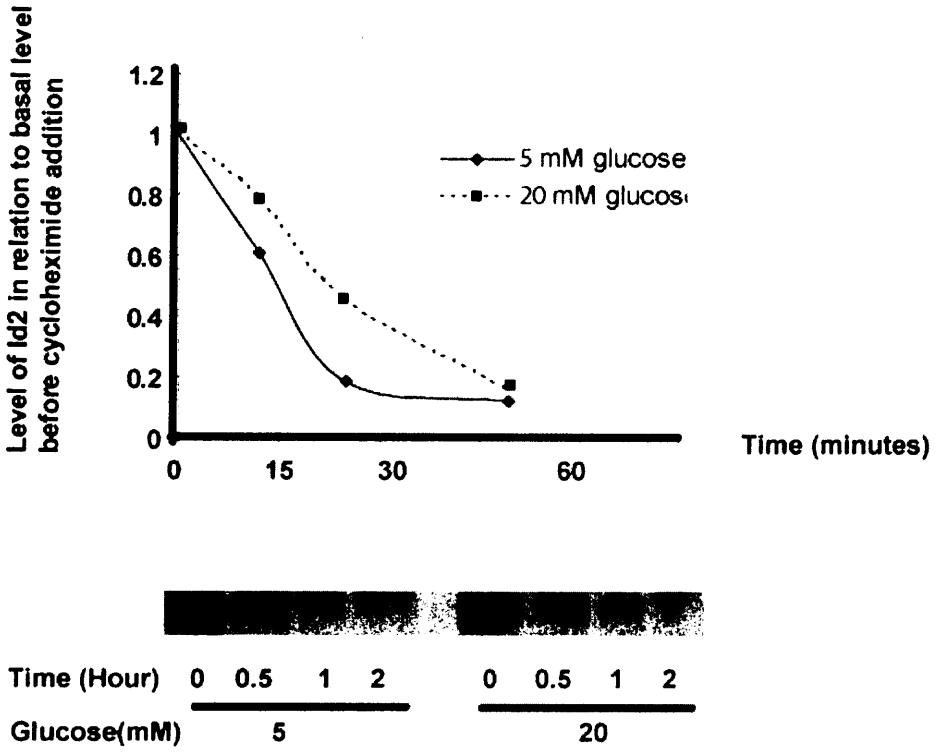


Figure 4.12 Cycloheximide analysis

J774.2 cells were incubated with 5mM glucose or 20 mM glucose for 6 hours before the addition of 10μM cycloheximide. Cells were lysed after 30 minutes, 60 minutes and 2 hour. Id2 were immunoprecipitated and the level analysed by western blotting. The graph represent quantitation of a representative experiment from three separate experiment. Data are represented as relative level compared to the original level before addition of cycloheximide.

Figure 4.13 Id2 in other cell type

L6 myocytes (A), rat soleus muscles (B), cardiomyocytes (C), HepG2 cells (D) and differentiated 3T3-L1 cells (E) were treated with 5mM or 20mM glucose or 2mM glucosamine as stated for 24 hours. Id2 were immunoprecipitated and the level analysed by western blotting. Quantitation of several experiments was performed for HepG2 and 3T3-L1 cells. Data represent the mean ± SE of four independent determinations performed in duplicate. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicate differences where P≤0.01.

(A) L6 myocytes

Glucose (mM)	5	20	5	5	5	5
Glucosamine (mM)	0	0	0.2	2	0	0
Insulin (uM)	0	0	0	0	0.1	1

(B) Soleus muscle

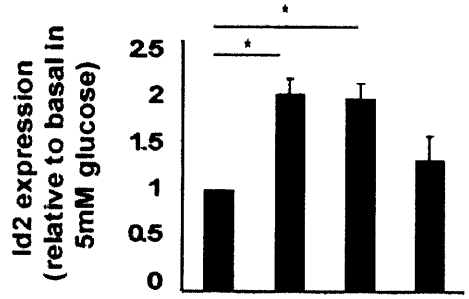
Glucose (mM)	5	20	5	20	5	5	20
Glutamine	-	-	+	+	-	-	+
Glucosamine (mM)	0	0	0	0	2	0	0
Insulin (uM)	0	0	0	0	0	1	1

(C) Cardiomyocytes

Glucose (mM)	5	20	5	5
Glucosamine (mM)	0	0	0.2	2

(D) HepG2 cells

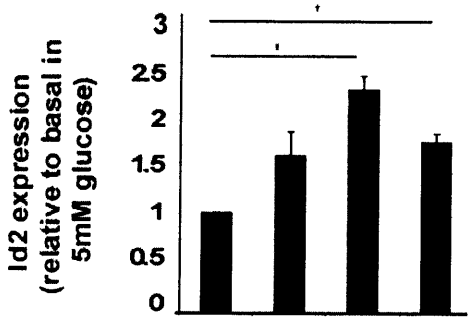
Glucose (mM)	5	20	5	5
Glucosamine(mM)	0	0	0.2	2



Glucose (mM)	5	20	5	5
Glucosamine(mM)	0	0	0.2	2

(E) Differentiated 3T3-L1 cells

Glucose (mM)	5	20	5	5
Glucosamine(mM)	0	0	0.2	2



Glucose (mM)	5	20	5	5
Glucosamine(mM)	0	0	0.2	2

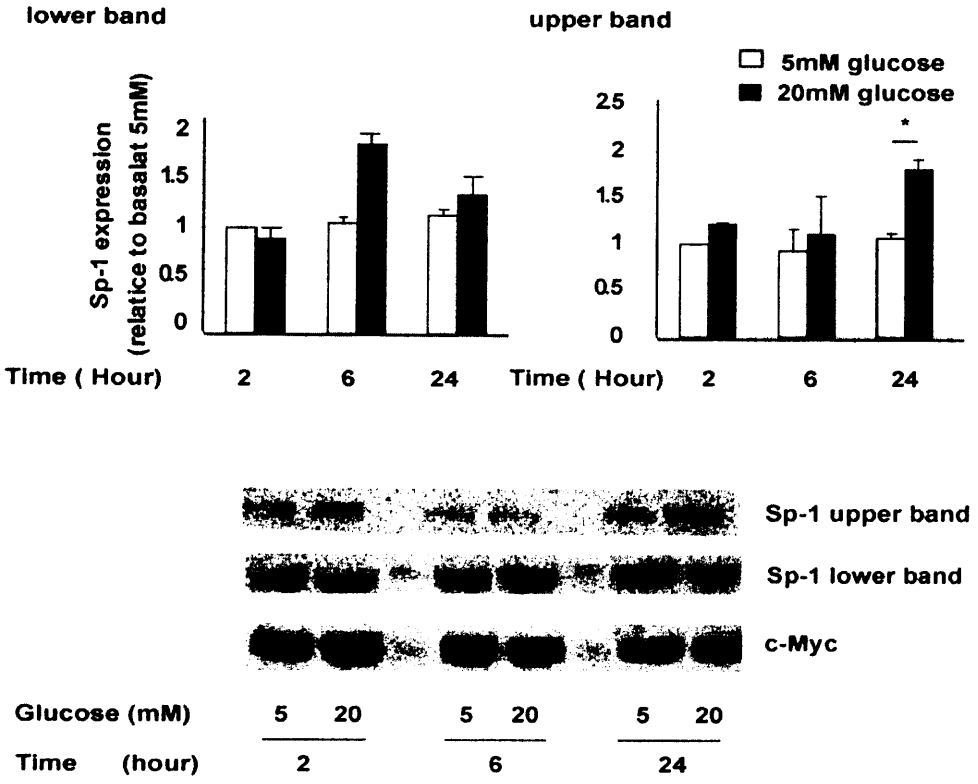


Figure 4.14 Level of Sp-1 increases in high glucose after 24 hours

J774.2 macrophages were incubated with RPMI 1640 containing 5mM and supplemented with 0.2% BSA overnight before cells were treated for further 2, 3 or 24 hours with 5mM or 20mM glucose. After the designated incubation time cells were lysed in RIPA buffer and samples separated on SDS-PAGE. Relative level of Sp-1 and Myc was determined by western blotting. Relative level of Sp-1 was quantified. Data represent the mean ± SEM of three independent experiments. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicates differences where P≤0.0.5.

4.3.13 Combined effects of glucose and hormones : Id2 level was maintained when leptin was combined with high glucose

Observations involving SREBP in the previous chapter raised a possibility that level of Id2 could be affected by a combination of hormone treatment and high glucose. There is a possibility that leptin together with glucose might alter Id2 level in an unforseen manner. To assess whether changes occurred, we pretreated J774.2 cells with 5mM or 20mM glucose overnight before treating them with leptin for 6 hours. As shown in Figure 4.15 the level of Id2 was not affected when leptin were combined with high glucose. After leptin treatment, Id2 level was maintained close to the level at high glucose alone. Leptin has no additive effect at high glucose.

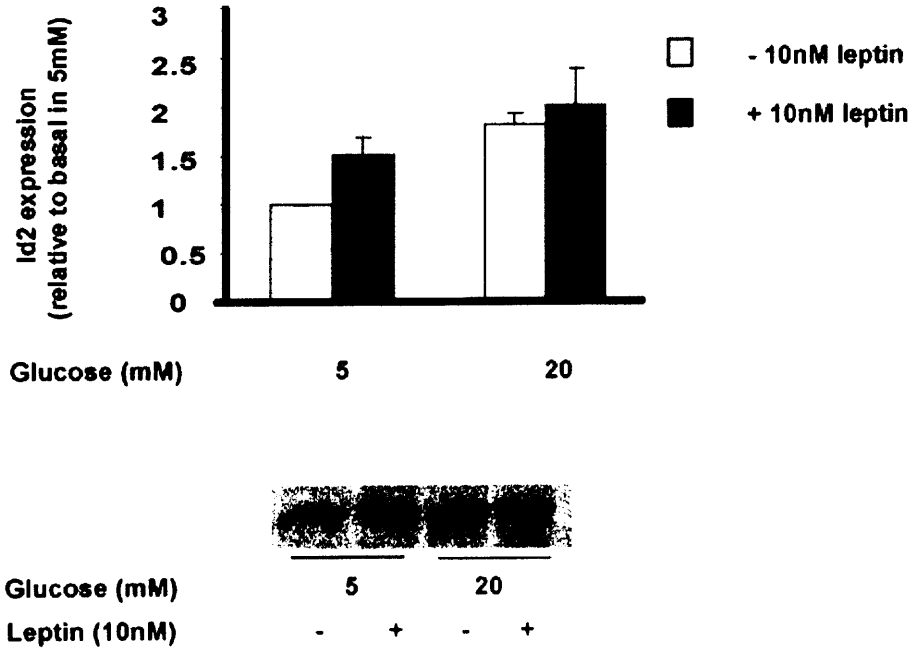


Figure 4.15 Co treatment with glucose and leptin does not change Id2 level

J774.2 cells were pre treated overnight in 5mM or 20mM glucose RPMI 1640 media. Subsequently cells were treated with 10nM leptin for 6 hours and extract prepared. Id2 was immunoprecipitated and its detected by western blotting. Relative level of Id2 was quantified. Data represents mean ± SEM of two experiments.

4.3.14 Id2 attenuates SREBP-1 mediated activation of HSL promotor

Previously Moldes et al demonstrated that Id2 is a binding partner of SREBP-1. Formation of Id2-SREBP-1 heterodimer inhibits DNA binding. Using FAS gene as a model of SREBP regulated promotor in 3T3-L1 adipocytes they have shown that Id2 attenuates SREBP transcriptional activity (Moldes 1999). To test whether activation of the HSL promoter by SREBP is attenuated by Id2, we co-transfected Id2 expression plasmid, SREBP expression plasmid and HSL-promoter luciferase construct into CHO-IR cells. The total amount of DNA was kept constant by the addition of empty expression plasmid. As shown in Figure 4.15, we found that co-transfection of Id2 significantly attenuated SREBP activation of HSL promoter. There was no effect on the empty plasmid control.

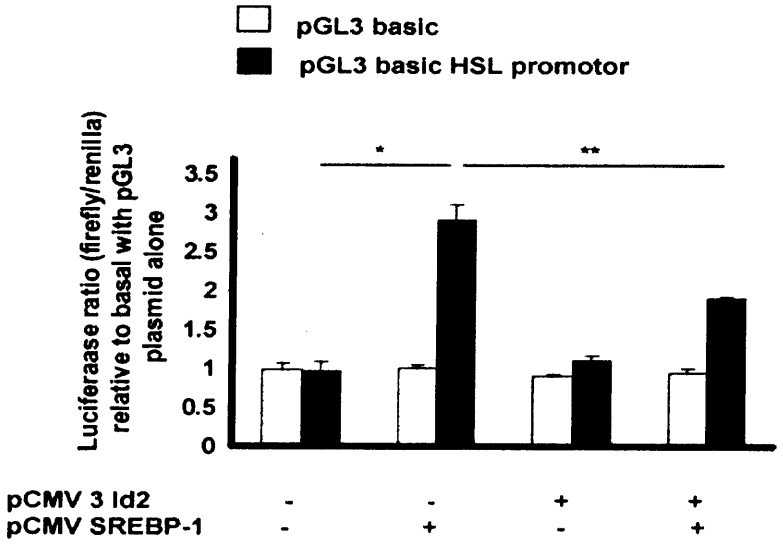


Figure 4.16 SREBP-1 co transfection increases HSL promotor activity and Id2 diminishes SREBP activation

CHO cells were split at 1 in 4 ratio in antibiotic-free media 24 hours before the start of transfection. Transfection was performed on roughly 50% confluent cells. Cells were transfected with pGL3 basic plasmid or pGL3 basic plasmid containing -2682 to + 301 residue or HSL promoter with or without pcDNA3 Id2 or pCMV SREBP-1 plasmids. Renilla pTK plasmid was used as an internal control and the total amount of DNA was kept constant by the addition of empty plasmid. The transfection reagent used was lipofectamine. After 24 hours, cells were lysed with luciferase assay buffer and reading taken by luminometer. The ratio between firefly and renilla was taken. Experiments were performed three times in triplicate. Data represent the mean ± SEM of a representative triplicate. Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicates differences where $P \leq 0.01$ and ** indicates $P \leq 0.05$.

4.4 Discussion

4.4.1 Summary of findings

Id2 is a well established controller of cell differentiation and cell cycle progression. These studies here identify Id2 as another transcription factor whose level is affected by changes in glucose concentration in J774.2 macrophages. These findings, combined with the fact that cAMP increases the level of Id2 (Moldes 1999) and its close relatives Id1 and Id3 are increased by glucose (Wice, Bernal-Mizrachi et al. 2001) and fatty acid (Busch, Cordery et al. 2002) suggests that Id proteins could play a wider role in cellular response to changes in nutrient status as its levels are responsive to changes in glucose concentration.

Growing evidence indicates that increased flux through hexosamine biosynthesis pathway is a major mediator of glucose effects on gene expression. An increasing number of mediators continue to be discovered. This study shows that Id2 is another protein affected by hexosamine flux. In support of the contribution of hexosamine flux to changes in Id2 level, we have shown that the changes were sensitive to azaserine, an inhibitor of GFAT. GFAT overexpression and glucosamine treatment increased level of Id2 while glutamine removal inhibited the glucose effect on Id2. By utilising mannitol, 3-o-methyl glucose and 2-deoxyglucose we have ruled out the involvement of osmotic stress, unmodified form of glucose or downstream glucose metabolite in the control of Id2 expression. Conventional PKC does not seem to be involved in the process since downregulation of PKC alpha and beta after chronic PMA treatment does not seem to affect the glucose effect on Id2 level. This observation does not rule out involvement of atypical or novel PKC . Furthermore, although PKC is downregulated, effects from the acute activation of PKC by PMA has been observed at a chronic time point.

In addition to changes in J774.2 macrophages, we found similar responses to glucose in HepG2 hepatoma cell line and in isolated soleus muscle. The changes were replicated by glucosamine addition in both cell types. Glucosamine also significantly increased Id2 expression in 3T3-L1 cells although the changes in response to glucose were not statistically significant. We found no response to glucose in L6 myoblast or isolated primary cardiomyocytes. Wide spread changes in level of Id2 in response to glucose suggest that this transcription factor may contribute to nutritional regulation of gene expression in many metabolically important tissues.

4.4.2 Hexosamine flux and changes in Id2 level The effects of glucose on Id2 levels could conceivably be mediated by changes in Id2 messenger RNA levels, changes in the rate of translation of this mRNA or changes in the stability of the Id2 protein. These possibilities are discussed below.

4.4.3 Mechanisms linking hexosamine flux to pre-transcriptional regulation

The Id2 promoter is the target for Myc (Lasorella 2000) and Sp1. The latter is known to regulate the expression of cell cycle regulator retinoblastoma (Datta, Raychaudhuri et al. 1995) as well as the expression of other transcription factors such as YY1 (Lee, Galvin et al. 1993) and E2F (Karlseder, Rotheneder et al. 1996). Sp1, a regulator of TATA-less promoter, is highly glycosylated in response to glucose (Du 2000). Glucosamine treatment and glycosylation increases the activity of its transcriptional activation domain (Goldberg, Whiteside et al. 2002). Additionally, o-linked glycosylation promotes its localisation to the nucleus (Brasse-Lagnel, Fairand et al. 2003). On the Myc oncoprotein, O-linked glycosylation is known to alternate with phosphorylation on Thr 48. Phosphorylation on this residue is important for Myc nuclear exclusion, hence it has been postulated that glycosylation could play a role in nuclear localisation and activation of Myc (Kamemura, Hayes et al. 2002).

Increased nuclear transport and transcriptional activity of these factors in response to O-linked modification could contribute to an increase in transcription from Id2 gene in response to increased hexosamine flux. Our result in Figure 4.14showed that in J774.2 macrophages, Sp1 levels increased after 24 hours incubation in high glucose. Such observation raises the possibility that an increase in Sp1 level in our system may contribute to an upregulation of Id2. While we have not carried out detailed analysis, to confirm this it would be necessary to assess the result of hexosamine flux on Id2 promoter attached to luciferase. Deletion studies should be carried out to assess contribution of each transcription factor binding site to Id2 expression. Subsequently, band shift assays should be performed and Myc and Sp1 could be co-transfected with Id2 promotor-luciferase and incubated in either low or high glucose conditions to assess their contribution to Id2 promotor activity.

4.4.4 Glucose and post-transcriptional regulation

Alternatively, glucose may affect the stability of Id2 mRNA without affecting rate of transcription. Depending on sequence and structure of each mRNA, glucose can potentially stabilise or destabilise the transcript (Patel, Eichler et al. 2003). Glucose stabilisation of mRNA is a common mechanism in yeast (Kim, Kim et al. 2002). It also contributes to stabilisation of insulin mRNA in pancreatic islet. In these cells glucose stimulates binding of polypyrimidine tract binding protein to a pyrimidine-rich sequence located in the 3'-

untranslated region of insulin mRNA, causing an increase in mRNA stability (Tillmar, Carlsson et al. 2002). Interestingly, recent evidence has shown that hexosamine flux mediates the positive effect of glucose on mRNA stability in the liver. An mRNA turnover study of ApoAI mRNA from HepG2 cells reveals that glucosamine treatment extends the half-life of ApoAI mRNA from 7 hours to 16 hours (Haas, Wong et al. 2004). Hexosamine flux could potentially exert a similar effect on Id2 mRNA in macrophages although this has not been investigated.

4.4.5 Glucose and regulation of translation

Alternatively, glucose could accelerate translation rate without changing the level of mRNA. In pancreatic β-cells, glucose accelerates the translation of preproinsulin through an element on the 5'-UTR, most likely to be a conserved stem loop secondary structure (Knight and Docherty 1992). Glucose also increases the overall rate of translation through protein phosphorylation by regulating the phosphorylation and hence activity of eukaryotic initiation factors (Rhoads 1999). Glucose increases activity of the guanine nucleotide exchange factor eIF-2B within 15 minutes of exposure in islets of Langerhans (Gilligan, Welsh et al. 1996). In β-cells, glucose also accelerates the elongation steps by accelerating the dephosphorylation of EF-2 factor via protein phosphatase-2A (Yan, Nairn et al. 2003).

Macrophages are another cell type with appropriate factors that allow for glucose regulation of translation rate. Glucose-stimulated changes in level of CD36 are the primary example (Griffin, Re et al. 2001). Increased protein levels occur as a result of enhanced translational efficiency in the presence of high glucose. These phenomena have been observed in macrophages differentiated from human peripheral blood monocytes as well as in macrophages obtained from human vascular lesions. Fractionation of polysome preparation derived from macrophages grown under high or low glucose on sucrose gradient followed by reverse transcriptase PCR on gradient fractions using primers specific for 18S or 28S shows that mRNA from high glucose preparation resides with the heavier fraction with more ribosomes. This demonstrates an increase in translation efficiency under high glucose for CD36 through greater ribosome loading. Features in the 5'-UTR such as length, secondary structure and existence of open reading frames contributes to this specificity. For CD36, mutagenesis and ribosome reinitiation studies have shown that the upstream open reading frame region in the 5'-untranslated region of CD36 mediates this glucose sensitivity by increasing the efficiency of reinitiation following translation. A similar experiment would reveal whether this is the mechanism underlying glucose-induced changes in Id2 protein level.

4.4.6 Hexosamine and the control of protein degradation

The current study investigated the effect on glucose on the post-translational stability of Id2. We found that high glucose slowed down the rate of Id2 protein degradation. This is likely to contribute to differences in level of Id2 in low glucose and high glucose, especially after chronic incubation. High glucose and subsequent modification of proteins with o-linked glycosylation are known to increase the half life of proteins through several mechanisms. On the very low density lipoprotein (VLDL) receptor, clusters of o-glycosylation prevent access of proteases to protease-sensitive sites on the receptor (Magrane, Casaroli-Marano et al. 1999). For the murine estrogen receptor beta, glycosylation seems to increase stability through its effect on PEST sequence-targetted degradation (Cheng and Hart 2001). Phosphorylation can change Ser or Thr residues into negatively charge residues. This can convert some imperfect PEST sequences into PEST degradation signal (Rechsteiner and Rogers 1996). Glycosylation alters this phosphorylation effect by competing at the same hydroxyl directly or by changing the protein conformation indirectly to mask the charged region.

For proteins which are degraded through ubiquitin-mediated proteasomal degradation such as Myc, o-linked glycosylation competes for the same residue as phosphorylation that directs the protein for ubiquitination. Conversely it increases stability of these proteins (Kamemura, Hayes et al. 2002). Glycosylation also works directly on the proteasome to prolong the half life of proteins. The 26S proteasome is a multi-subunit complex that catalyses ATP-dependent degradation of proteins. It is made up of 20S catalytic complex and 19S regulatory subunit. Recognition of ubiquitinated substrate by the 19S unit is followed by deubiquitination and protein unfolding. The denatured polypeptide is then degraded by proteases on the 20S unit. When the 19S unit is glycosylated under high glucose condition, its ATPase activity is diminished. Consequently, the rate of proteasome-mediated degradation decreases, leading to an increase in stability of many proteins. It is very likely that a slower rate of proteasome complex contributes to an increase in stability of Id2. At this point it is unclear whether competition between phosphorylation and glycosylation contributes to Id2 stability. In a cell-free system phosphorylation does not seem necessary for the targetting of ubiquitin to Id2 (Fajerman, Schwartz et al. 2004) but in a cell line, Id2 levels decrease after phosphorylation on Ser 5 by CDK2 (Hara 1997).

4.4.7.1 Glycosylation and implications on Id2 functions: intermolecular interaction

Ample evidence demonstrates that o-linked glycosylation alters intermolecular interactions. The impact on intermolecular interaction, however, could be either negative or positive depending on the surrounding environment. Work in synthetic peptides and molecular dynamic simulation shows that o-glycosylation can exert a stabilising effect on the secondary

structure of designed alpha-loop-alpha motif by decreasing the opportunity for water molecules to compete for intramolecular hydrogen bond (Liu, Hwang et al. 2004). Conversely, on synthetic peptides with hairpin helix loop helix motifs that dimerise to form four helix bundles, glycosylation reduces the helical content of the polypeptides and increases instability of the helix-loop-helix dimers. This shifts the balance toward monomers formation (Vijayalekshmi, George et al. 2003).

The situation is similar in physiological proteins. On p53, o-linked glycosylation disrupts intramolecular interaction of inhibitory domain, allowing DNA binding (Shaw, Freeman et al. 1996). Disruption of intramolecular interactions underlines the proposed role of OGT as a global transcriptional repressor (Yang, Zhang et al. 2002). Conversely, on STAT5 o-linked glycosylation increases its interaction with CBP protein (Gewinner, Hart et al. 2004) while glycosylation of Elf1(Juang, Solomou et al. 2002) and YY1 (Ivins, Pemberton et al. 2003) disrupt interaction with inhibitory protein and increase their DNA binding.

The impact of o-linked glycosylation on intramolecular reactions may play a vital role in the function of Id2 since it has been known to exert its effect by dimerising with bHLH factors. Observations made using synthetic peptide containing helix loop helix motifs (Vijayalekshmi, George et al. 2003) suggest that glycosylation might disrupt the binding of Id2 to other factors, however it is premature to make this conclusion since side effects from surrounding residue could reverse the effect. To resolve this will require approaches such as mutagenesis and co-immunoprecipitation studies.

4.4.7.2 Glycosylation and implications on Id2 function: nuclear import

Present evidence tends to link glycosylation to nuclear import of proteins such as Tau (Lefebvre, Ferreira et al. 2003) and Sp1(Brasse-Lagnel, Fairand et al. 2003). Whether it affects localisation of Id2 remains to be seen. Although Id2 is small enough to diffuse passively through the nuclear pore, its location is tightly controlled in response to differentiation and cell cycle stages (Tu, Baffa et al. 2003). Nuclear shuttling is an important mechanism controlling activity of Id2 (Kurooka and Yokota 2005). The basic helix-loop-helix domain contributes to the nuclear import of Id2 while the C-terminus leucine-rich nuclear export signal interacts with nuclear export receptor chromosome region maintenance protein-1. Modification of these residues by glycosylation may disrupt interaction of helix loop helix domain or the C-terminal domain with respective binding partners.

4.4.7.3 Glycosylation and implications on Id2 function: phosphorylation of Id2

Id2 is a substrate of cyclin E-Cdk2 and cyclin A-Cdk2. Phosphorylation of a serine residue 5 within consensus Cdk site prevents Id2 from interfering with the formation of E-box DNA binding complexes *in vitro*. In human diploid fibroblasts phosphorylation of Id2 occurs after 12 hours and is maintained to 24 hours. This coincides with late G1 phase and activation of cyclin E-Cdk2 and the formation of E box DNA binding complex (Hara 1997). It has been postulated that this phosphorylation alters binding specificity of Id2. While in early G1, Id2 binds mainly to E2A like basic helix-loop-helix protein, phosphorylation may change its preference to retinoblastoma in late G1. Ser 5 phosphorylation also plays a role in Id2 nuclear translocation. Mutant Id2 lacking the phosphorylation residue tends to accumulate in the cytosol instead of the nucleus (Matsumura, Lobe et al. 2002). Replacement of phosphorylation with glycosylation in this case could affect the balance between cell cycle progression and differentiation.

Another experiment *in vitro* demonstrates that Id2 can be phosphorylated by PKA and protein kinase C alpha. The phosphorylation sites lie within the helix loop helix motif, but neither phosphorylation affects the dimer formation between Id2 and E47 helix loop helix factor (Nagata 1995). Instead, this phosphorylation might affect Id2 translocation or its interaction with other unidentified partners in the cytosol.

4.4.7.4 Glycosylation and implications on Id2 function : alteration in intracellular targeting

A recent study on membrane type 1 matrix metalloproteinase (MT1-MMP) shows that o-linked glycosylation is involved in intracellular targetting. The mutated and unglycosylated form of the enzyme is active and oligomerises normally, however it fails to transport TIMP-2, a component of the active complex, to cell surface. This causes defective formation of the MT1-MMP/TIMP2-proMMP2 complex that catalyses the maturation of pro matrixmetalloproteinases-2. The exact mechanism underlying this disruption is unclear but an unidentified carbohydrate binding scaffold protein might stabilise the active confirmation of the enzyme, allowing the interaction with TIMP-2 protein. There are possibilities that carbohydrate binding scaffold proteins may bind to glycosylated form of Id2 and direct its intracellular targeting. However this hypothesis remains to be studied.

4.4.8.1 Functional consideration : Id2 and lipid metabolism

We have shown that accumulation of Id2 was maintained when chronic hyperglycaemia was combined with hormones. Slower rate of protein degradation could be responsible for the accumulation. Subsequently we have confirmed that Id2 coexpression reduced SREBP

dependent activation of the HSL promoter. Together both observations suggest that accumulation of Id2 may contribute to reduced HSL expression in J774.2 macrophages treated with chronic hyperglycaemia and hormones. Pools of accumulated Id2 in the cytosol and the nucleus may interact randomly with its partners, causing various pathological developments including dysregulation of lipid metabolism. Modification of Id2 by glycosylation may enhance this interaction further, allowing it to sequester any free SREBP available. The interaction may occur both in the nucleus and the cytoplasm since the site of interaction between SREBP and Id2 has not been elucidated.

Given the role of Id2 in lipid regulation, it would be very interesting to analyse the lipid profile of Id2 knockout mice. So far it has been confirmed that Id2 and ApoE double knock out mice are more resistant to atherosclerosis than Apo E knockout mice alone (Aoki 2003). It would be interesting to assess the activity levels of various metabolic enzymes in macrophages, liver, muscle and fat of Id2 knockout mice. Generation of mice containing macrophages with Id2 specifically knocked out in macrophages could be created by bone marrow transplantation and may reveal further contributions of this factor to lipid metabolism in immune cells.

4.4.8 .2 Functional consideration : Id2 and cancer

In addition to cardiovascular disease, several cohort studies have demonstrated that diabetes is associated with an excess risk for all cancers (Levine, Dyer et al. 1990; Steenland, Nowlin et al. 1995; Eschwege, Charles et al. 2001). Tissue-specific studies have shown the link between diabetes and cancer in the pancreas (Everhart and Wright 1995), liver (Adami, Chow et al. 1996), and colorectal cancer (Hu, Manson et al. 1999). Hyperglycaemia, glucose intolerance, elevated fasting and postpradial glucose have been associated directly with risk of cancer development (Jee, Ohrr et al. 2005) (Chang and Ulrich 2003). The underlying mechanism linking both factors remains a mystery. It has been suggested that anaerobic metabolism in cancerous cells may benefit from hyperglycaemia as more abundant energy source is available (Jass 1985), but an improvement in our understanding of cellular processes continue to suggest novel contributing factors. Evidence suggests that increased flux through hexosamine pathway might contribute to tumour development.

Oncogenes such as p53, Myc and HIC1 (Lefebvre, Pinte et al. 2004) are modified by o-linked glycosylation. Accumulating evidence documents the involvement of complex glycan on cell surface glycoprotein in tumour progression and mice lacking N-acetyl glucosaminyltransferase III show retarded liver tumour progression (Yang, Tang et al. 2003). There is also evidence for changes in activity of hexosamine pathway enzymes in cancer cells.

Activity of L-glutamine-fructose-6-phosphate amidotransferase (glucosamine-6-phosphate synthase), which catalyses the formation of glucosamine-6-phosphate (Milewski 2002), increases in neoplastic liver tissues (Kikuchi, Kobayashi et al. 1971) and in actively proliferating liver tissue such as liver of partially hepatotectomised rat (Akamatsu and Maeda 1971).

Our observation suggests that in addition to the changes mentioned above, the accumulation of Id2 in response to increased hexosamine flux under chronic hyperglycaemia could be another underlying reason of increased cancer risk in diabetic patients and in patients with impaired glucose tolerance.

Id2 can be recognised as oncogene in its own right. Given the role of Id2 in the control of proliferation in many cell types, it seems likely that expression of abnormal amount of Id2 in tumour cells would lead to excessive cell growth. Studies have shown that Id2 plays important role in the initiation, growth and angiogenesis of pituitary tumours in Rb(+/-) mice (Lasorella, Rothschild et al. 2005). Id2 knock out mice have reduced tumour size and proliferation rate (Lasorella, Rothschild et al. 2005). Its expression has been correlated with mitotic index of human primary adenocarcinoma and it is upregulated in intestinal mucosa of p53 null mice (Wilson, Deed et al. 2001). Activation of beta-catenin/TCF pathway has been linked to Id2 up regulation in colon carcinoma and consequently there is an increase in anchorage independent survival of these cells (Rockman, Currie et al. 2001). Overexpression of Id2 induces characteristics of transformation sin NIH 3T3 fibroblasts such as ability to grow on soft agar and it is required for malignant behaviour of neuroblastoma cells (Lasorella, Boldrini et al. 2002). Targetted expression of Id2 in T-cells generated aggressive thymic lymphomas which rapidly kill the transgenic mouse (Morrow, Mayer et al. 1999).

In addition to experiments involving cell lines and transgenic mouse manipulation, accumulation of Id2 has been detected in primary human cancer. Unusual overexpression has been detected in pancreatic tumours(Kleeff, Ishiwata et al. 1998) and in tissue section of squamous cell carcinoma (Langlands, Down et al. 2000). It is a target of EWS/Ets protein in Ewing sarcoma (Fukuma, Okita et al. 2003) and is highly expressed in high grade astrocytes tumour (Vandeputte, Troost et al. 2002).

Tumour cells use various strategies to disrupt pathway leading to terminal differentiation, a process known as anaplasia. This is associated with ability to constitutively activate growth signalling (Evan and Vousden 2001). Accumulation of Id2 contributes to this process by sequestering retinoblastoma protein, allowing E2F1-3 helix loop helix factor to activate G1-S

phase progression (Lasorella 2000). Id protein also inhibits the activity of Ets factors, the activator of tumour suppressor p16Ink4a (Ohtani, Zebedee et al. 2001), further contributing to cell cycle progression. Another hallmark of cancerous cells is adaptive changes that allow them to survive and proliferate under hypoxic condition created by high growth rate that outpace angiogenesis (Harris 2002). Hypoxic tumours are more malignant and metastasise more frequently than oxygenated ones (Hockel, Schlenger et al. 1996). Hypoxia inducible factor-1 and -2 is a mediator of a transcriptional response pathway that allows cancer cells to survive hypoxia. One of the genes activated by these factors is Id2 (Lofstedt, Jogi et al. 2004). Activation of Id2 in hypoxic neuroblastoma cells could be linked to down regulation of neuronal lineage specification factors such as dHAND, HASH-1 and E2-2 (Jogi, Vallon-Christersson et al. 2004). It also sequests binding partners of HASH-1 and dHAND (Jogi, Persson et al. 2002), causing dedifferentiation and accelerated growth (Jogi, Ora et al. 2002)of hypoxic neuroblastoma cells.

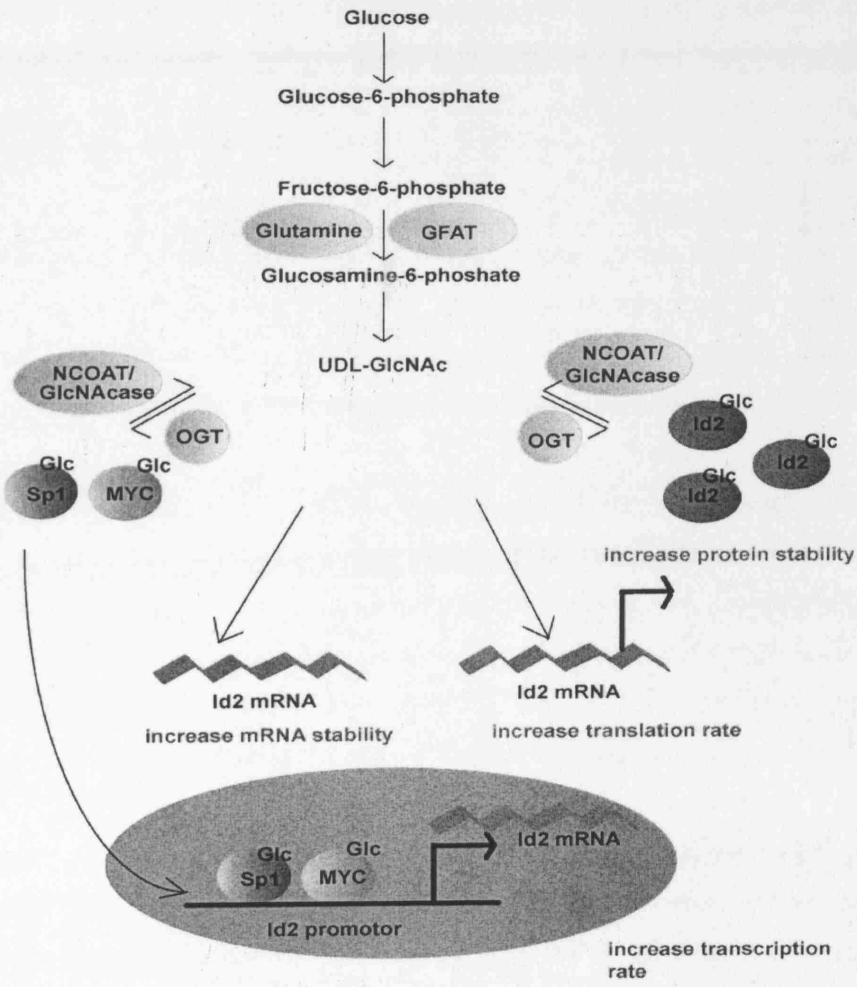


Figure 4.17 Summary diagram O-linked glycosylation prolongs the half life of Id2 protein, or other proteins such as Myc and Sp-1, which activate Id2 expression.

4.5 Conclusion

In conclusion, this chapter provides evidence that hexosamine pathway flux mediates the glucose effect on Id2 levels and Id2 itself is modified by o-linked glycosylation. Increases in Id2 levels can attenuate SREBP mediated activation of lipid homeostasis genes such as HSL. The chapter discusses the implications these findings might have for oncogenesis where many tumours have a high flux through the glycolytic pathway.

Chapter 5 Results

Effect of hormones on Id2 expression

5.1 Abstract

Our work in previous chapters focused on the effects of glucose on Id2 expression but there is also a great deal of evidence indicating that Id2 expression in many cell types is under tight hormonal control. The impact of various hormones on Id2 expression on macrophages has not been investigated. This chapter describes the effect of a range of hormones on Id2 expression in macrophages and the mechanisms employed.

We first studied cAMP, a secondary messenger generated subsequent to binding of certain hormones to cell surface receptors, as this is a strong inducer of Id2 expression in other cell types including differentiated 3T3-L1 and Sertoli cells. We found that 8-CPT-cAMP, a cell permeable analogue of cAMP, exerted a similar effect in J774.2 murine macrophages. Intracellular cAMP raising agents such as LPA, IBMX, adrenaline and leptin replicated the effect of 8-CPT-cAMP. The effect was PKA-independent. Instead, it was mediated by Epac as confirmed by a newly synthesized analogue of cAMP called 8-CPT-2Me-cAMP that specifically recognizes the novel cAMP effector Epac. Using various inhibitors of signalling pathways, we found that the effect required PI3-kinase activity but not ERK activity. Consistent with this there was an increase in PKB phosphorylation following administration of 8-CPT-cAMP, concomitantly with a reduction in Erk phosphorylation.

Another hormone of interest here is insulin. Previous work in the lab has shown that J774.2 cells possess a functional insulin signalling cascade (O'Rourke 2001). Since insulin-like growth factor has been identified as a strong inducer of Id2 expression, we studied the effect of insulin on Id2 level in J774.2 macrophages as well as in other insulin-sensitive tissues. We found that insulin raised the relative amount of Id2 protein in macrophages, hepatocyte, adipocytes and muscle cell through a PI3-kinase dependent mechanism.

The involvement of PI3-kinase in both cAMP and insulin effect on Id2 expression prompted an investigation into a common downstream effector. The candidate focussed on was GSK3, as this is a downstream substrate of PKB and is involved in the control of transcription factors. We found that in J774.2 cells, insulin, 8-CPT-cAMP and 8-CPT-2Me-cAMP (the Epac activator) increased GSK3 inhibitory phosphorylation in a wortmannin-sensitive manner. Subsequently, direct links between GSK3 inhibition and an increase in Id2 protein

expression were confirmed using two different GSK3 specific inhibitors. In conclusion, we propose that GSK3 plays a vital role in the determination of Id2 protein level through a novel pathway that proceeds through cAMP --- Epac --- Rap-1--- PI3 kinase --- PKB --- GSK3 or insulin---PI3kinase---PKB---GSK3 , resulting in the upregulation of Id2 protein level.

5.2 Introduction

Many epidemiological studies reveal that abnormal levels of hormones associated with diabetes can be correlated to accelerated atherosclerotic lesion progression. In order to understand lesion development, it is vital to study hormonal effects on gene expression in various cells around the lesion area. Knockout mouse models are a useful tool for the identification of candidate genes that may contribute to atherogenesis. Recently, the Id2 knockout model has revealed that Id2 is another potential contributor to atherosclerotic progression. In the Id2/ApoE double knockout mouse, lesion development is significantly diminished compared to the ApoE knock out mouse (Aoki 2003). Despite the fact that Id2 could play a vital role in atherogenesis and that the macrophage is one of the main targets of hormonal actions, nothing is known about the effect of various hormones on Id2 level in macrophages. Here we attempt to explore the effect of diverse hormones on Id2 expression in macrophages using J774.2 murine macrophages as a model.

Expression of Id2 has been studied extensively in Sertoli cells and 3T3-L1 adipocytes. In these cells, hormone or agents that raise intracellular cAMP act as strong inducers of Id2 expression. Id2 mRNA starts to increase after 2 hour, reaching its maximum level after 6 hours and decreases down to basal level after 24 hours(Scobey 2004). Cyclic-AMP is a well-established second messenger regulating a wide variety of cellular processes in macrophages. It controls cytokine synthesis, phagocytosis, adhesiveness and NO generation. There are possibilities that cAMP may exert a similar effect on Id2 protein expression in macrophages. This study, therefore, aims to assess whether cAMP affects Id2 protein levels in J774.2 cells.

The effects of cyclic AMP are traditionally thought of as being propagated through protein kinase A. Binding of cAMP causes dissociation of the catalytic subunit from the regulatory subunits. Unbound catalytic subunits phosphorylate a wide array of intracellular targets, including CREB which mediates a sizeable part of the effects on gene expression. However, we now know that PKA does not account for every intracellular action of cAMP. In 1998, Bos et al cloned an exchange protein with cAMP-binding domain similar to the cAMP binding domain in the regulatory subunit of PKA and named it "exchange protein directly activated by cAMP 1" (Epac1). Both Epac1 and Epac2 act as GTP exchange factors for the small GTPase Rap1 and Rap2. Recently, a study by Aronoff et al (Aronoff, Canetti et al. 2005) shows that Epac is expressed in human and rat alveolar macrophages and in RAW264.7 cells. Epac mediated the cAMP-dependent inhibition of phagocytosis and hydrogen peroxide production in these cells. We found that Epac is expressed in human monocyte-derived macrophages and J774.2 cells (L. Gronning, personal communication),

therefore mechanistic studies must take into consideration both PKA-dependent and independent pathways.

Regulation of Id2 expression also occurs in response to extracellular growth factors and mitogens in many cell types with maximum response around 1-3 hours similar to other early response genes (Christy 1991; Biggs 1992; Deed, Bianchi et al. 1993; Barone 1994; Hara 1994; Tournay 1996). In 32D haematopoietic cells, Id2 level increases with stimulation by insulin-like growth factor-1 (IGF-1), a signal involved in proliferation and differentiation of cells, in a PI3-kinase dependent manner (Barone 1994; Baudino and Cleveland 2001; Belletti 2001; Navarro 2001; Prisco 2001). IGF-1 activates a similar signalling cascade to insulin, which is a hormone of great interest in out study since hyperinsulinaemia often accompanies diabetes and the condition correlates well with higher risk of cardiovascular disease development. Previously we found that J774.2 is an insulin-responsive cell type. The insulin signalling cascade in J774.2 macrophages is similar to the well characterised pathway (O'Rourke 2001). Insulin affects metabolism and function of macrophages in many ways. It increases phagocytosis and metabolism of glucose (Costa Rosa, Safi et al. 1996), while chronic hyperinsulinaemia in combination with high glucose reduces HSL expression and activity in J774.2 macrophages (O'Rourke 2002). Because of its role in macrophages function and the possibility that insulin might affect Id2 expression in a similar manner to IGF, we decided to investigate the effect of insulin on Id2 expression in J774.2 cells.

5.3 Results

5.3.1 8-CPT-cAMP increases Id2 protein level in J774.2 macrophages

cAMP and intracellular cAMP raising agents are known to be potent inducers of Id2 in 3T3-L1 and Sertoli cells. To investigate whether the same effect occurs in J774.2 cells, we used a cell-permeable analogue cAMP to generate a non-localised increase in intracellular cAMP. Confluent J774.2 macrophages were starved overnight in serum-free media containing 5mM glucose. After 18 hours cells were treated with 100, 200 and 400 μM 8-CPT-cAMP for 2 and 6 hours. The range of concentration used in this experiment was applied to J774.2 previously by Bernard et al (Bernard, Rodriguez et al. 1991).100μM of 8-CPT-cAMP induces the maximum response at both 2 and 6 hours. After 2 hours, the relative protein level of Id2 increases up to 2.7 fold at 100μM 8-CPT-cAMP (Figure 5.1).

5.3.2 Intracellular cAMP raising agents increase Id2 protein level in J774.2 macrophages

To assess whether endogenously generated cAMP can mimic the effect of the cell permeable analogues, J774.2 cells were starved in serum-free media overnight before being treated with a range of cAMP raising agents for 2 hours. Adrenaline was chosen as J774 cells are known to express β-adrenergic receptosr (Chambaut-Guerin and Thomopoulos 1987) and adrenaline is known to raise intracellular cAMP in J774.2 macrophages (Pang and Hoult 1997). We also used the broad spectrum phosphodiesterase inhibitor IBMX as blocking phosphodiesterases will raise basal levels of cAMP. In the present study, IBMX and adrenaline raised Id2 protein level after 2 hours treatment, although the amplitude is significantly less than those achieved with 8-CPT-cAMP as shown in Figure 5.2. Nevertheless, the observation confirms that cAMP generated intracellularly can affect Id2 protein expression in J774.2 macrophages.

We find that lysophosphatidic acid (LPA) and Leptin also act as intracellular cAMP raising agents in J774.2 macrophages (L. Gronning's personal communication). We decided to assess changes in Id2 expression after LPA treatment. The concentration used by W. Lin in RAW 264.7 cells was referred to as a guideline (Lin, Chang et al. 1999). In the present study, 25 μM and 50 μM of lysophosphatidic acid induced a small but significant increase in Id2 protein expression. We hypothesised that one reason that the increase may not be as large as seen with cAMP analogues could be that endogenously produced cAMP is continually being turned over by phosphodiesterases. We therefore looked to see whether IBMX would amplify the effects of LPA and found that the effect of LPA was synergistic with IBMX. When added together, induction of Id2 level occurred at a higher level than the effect of

either on its own. The synergistic effect was comparable in magnitude to the effect of 8-CPT-cAMP as shown in Figure 5.3. A lesser response occurs with leptin but a comparatively low concentration has been used in the present study.

5.3.3 The induction of Id2 protein level by 8-cpt-cAMP is not mediated by the PKA/CREB pathway

Because the effect of FSH on Id2 in Sertoli cells can be replicated by overexpression of PKA catalytic units (Scobey, Fix et al. 2004), we attempted to determine whether PKA is involved with the increase in Id2 protein level in J774.2 cells. We decided to use H89, an effective *in vivo* inhibitor of PKA, to assess the contribution of PKA. Suitability of H89 as specific inhibitor of PKA has been under scrutiny recently. The Cohen group has demonstrated that at the widely used concentration of 10 μM, H89 also inhibits S6K1, MSK1, ROCKII, PKB and MAPKAP-K1b (Davies, Reddy et al. 2000). The IC_{50} value for PKA is 135 nM and the value for PKB is 2.6 μM. We decided to look for the minimum amount of H89 required for PKA inhibition in J774.2 cells to minimise cross-inhibitory effect. By using CREB phosphorylation as the indicator of PKA activation, we assessed the minimum amount of H89 required for PKA inhibition in our system. Contrary to previously published literature, which utilises 20 μM (Mead, Hughes et al. 2003) or 50 μM (Tang, Vaughan et al. 2004) in J774 macrophages, we found that PKA activity and CREB phosphorylation were inhibited completely by 1μM H89. The inhibitory effect was maintained at 10 μM in our system as shown in Figure 5.4.

Having determined the suitable amount of H89, we next assessed for changes in Id2 level in response to 8-CPT-cAMP treatment with or without H89. Treatment with 1 μM or 10 μM of H89 for 30 minutes prior to the treatment with 100 μM of 8-CPT-cAMP did not block the 8-CPT-cAMP-induced increase in Id2 protein levels. This provides evidence that PKA does not participate in the cAMP signalling cascade leading to an increase in Id2 level in our system.

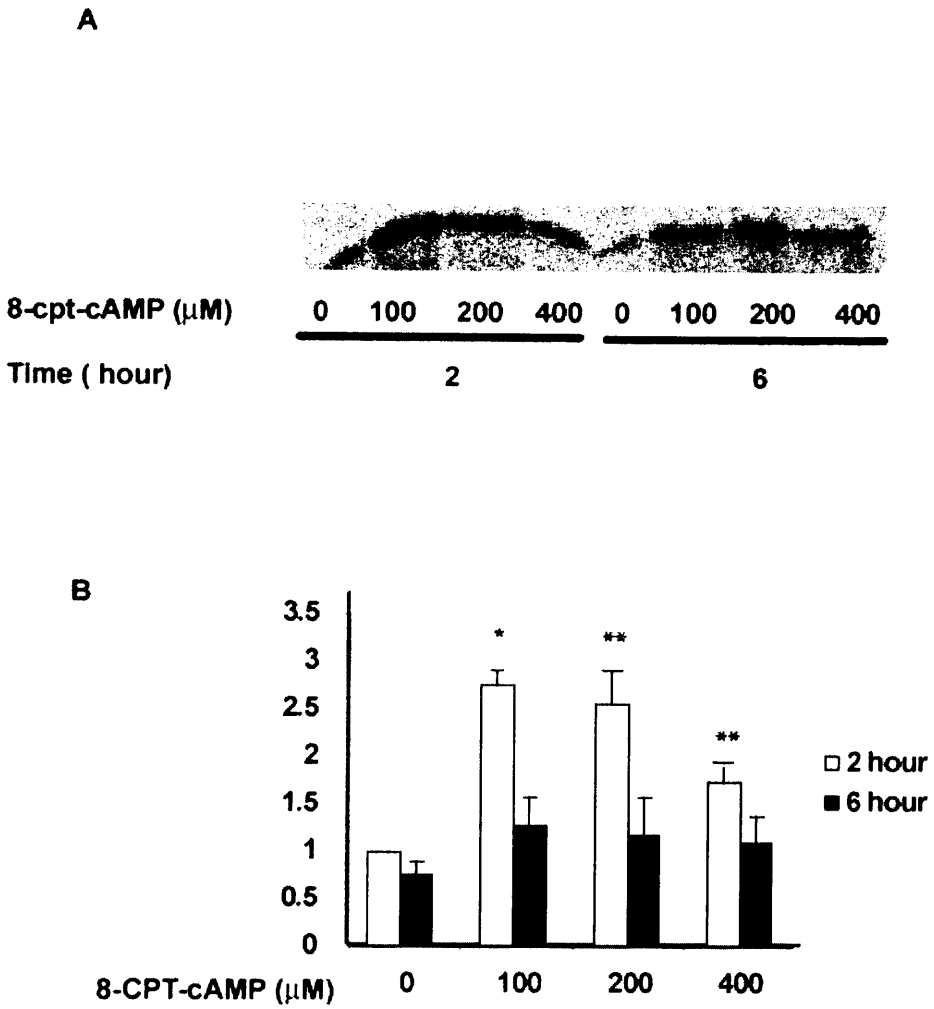


Figure 5.1 8-CPT-cAMP induces expression of Id2 protein

A: J774.2 macrophages were starved overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours cells were treated with 100, 200 or 400 μM of 8-CPT-cAMP for 2 or 6 hours. Id2 was immunoprecipitated and relative Id2 protein level was determined by Western blotting. B: Quantitation of three individual experiments (mean +/- SEM). Statistical analysis was performed using Student's paired t test. Significant differences are indicated where * indicates where $p<0.01$ and ** indicates where $p<0.05$.

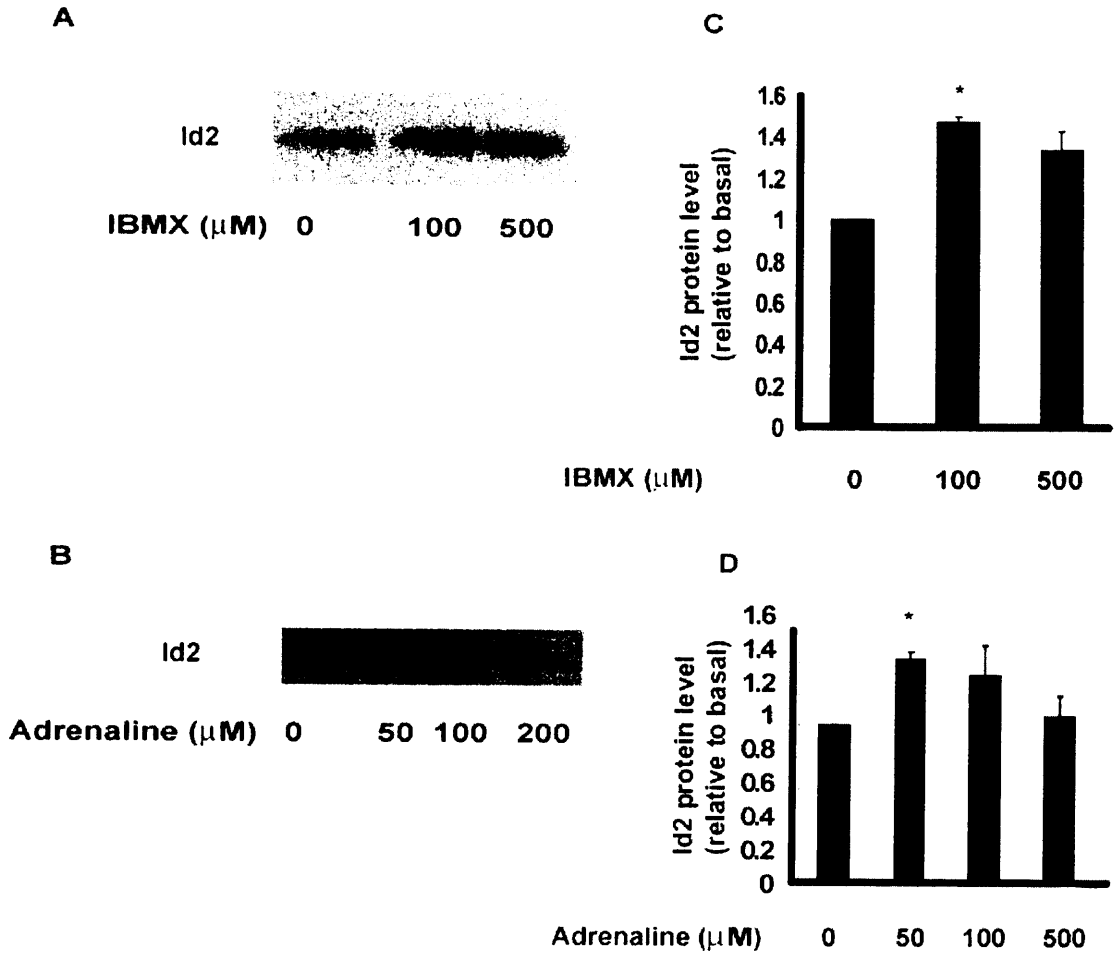


Figure 5.2 Adrenaline and IBMX increase protein level of Id2 in J774.2 macrophages

J774.2 macrophages were incubated overnight in serum-free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours cells were treated with 50,100 or 200 μM of Adrenaline for 2 hours (A) or 100 or 500 μM IBMX for 2 hours (B). Id2 was immunoprecipitated and relative Id2 protein level was determined by Western blotting. (D, E) Quantitation of (A, B) in successive order. Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using student's paired t test. Significant differences are indicated where * indicates where $p< 0.01$.

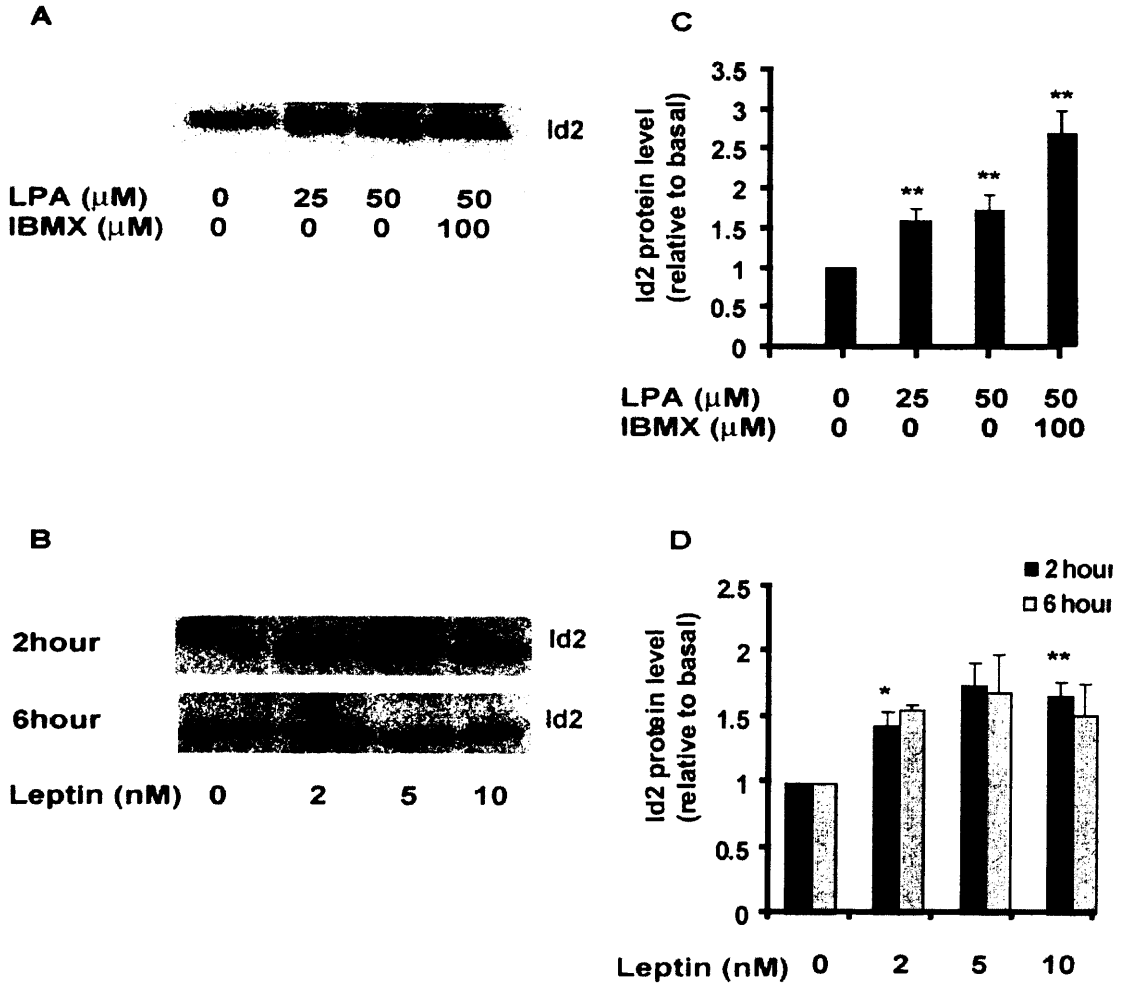


Figure 5.3 LPA and leptin increase protein level of Id2 while LPS does not affect the basal level

J774.2 macrophages were incubated overnight in serum-free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours cells were treated with LPA, LPS or a combination of LPA and IBMX for 2 hours (A). Otherwise, J774.2 macrophages were treated with 2, 5 or 10nM of leptin for 2 or 6 hours (B). Id2 was immunoprecipitated and relative Id2 protein level was determined by Western blotting. (C, D) Quantitation of (A, B) in successive order. Data represent mean ± SEM of three independent experiments. Statistical analysis was performed using student's paired t test. Significant differences are indicated where * indicates $p<0.01$ and ** indicates where $p<0.05$.

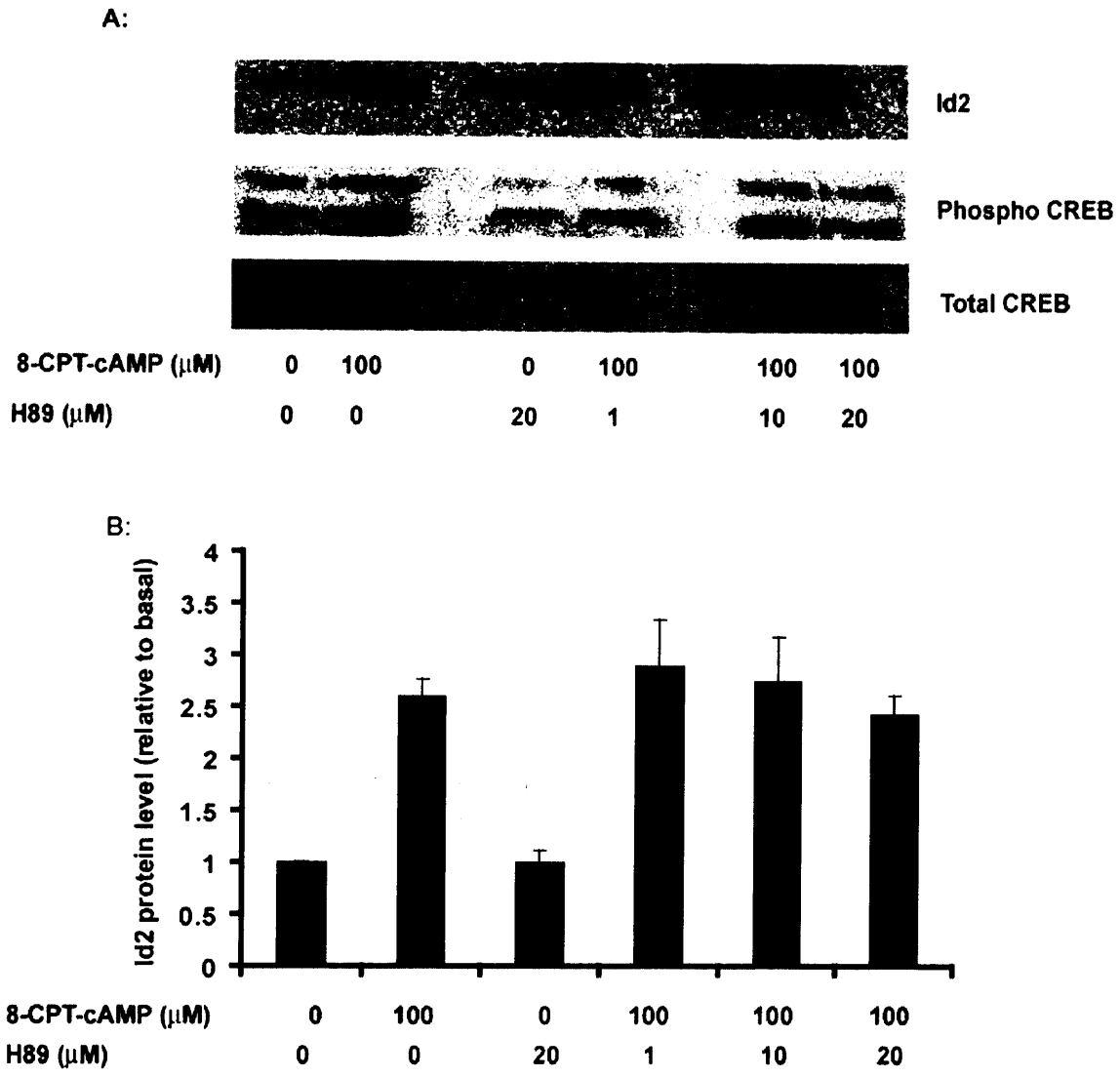


Figure 5.4 The effect of 8-CPT-cAMP on Id2 protein level in J774.2 macrophages is PKA independent

A:J774.2 macrophages were incubated overnight in serum-free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours cells were treated with H89 for 60 minutes before 100μM of 8-CPT-cAMP were added to the media. Id2 was immunoprecipitated after 2 hours and relative Id2 protein level was determined by Western blotting. Relative amount of Ser-133 phosphorylated CREB and total CREB were determined by western blotting. B: Quantitation of id2 level from three separate experiments.

5.3.4 Activation of EPAC mediates the increase in Id2 protein level

Having concluded that the increase in Id2 levels was PKA-independent, we explored the possibility that the effect could be mediated through Epac, a newly discovered cAMP effector that is a guanine nucleotide exchanger for RAP. To determine whether direct binding of cAMP to Epac was involved with the control of Id2 level, we employed 8-(4-chloro-phenylthio)-2-O-methyladenosine-3',5'-cyclic adenosinemonophosphate (8-CPT-2Me-cAMP), a newly characterised cAMP analogue, which specifically activates Epac but not PKA both *in vitro* and *in vivo*(Enserink, Christensen et al. 2002). The cAMP-binding domain of Epac lacks the highly conserved glutamine residue that forms hydrogen bonds with the 2'-hydroxyl of the cAMP ribose group. Consequently, the 2'-hydroxyl group on cAMP, which is absolutely required for efficient binding and activation of PKA, is not required for efficient activation of Epac. After the removal of 2'-hydroxyl group, 8-CPT-2Me-cAMP becomes a potent activator of Epac and its ability to activate PKA holoenzyme is greatly impaired.

To assess whether EPAC mediates the cAMP effect in our system, J774.2 cells were serum-starved in 5mM glucose media before being treated with 30 μM or 100 μM of Epac-specific activator, 8-CPT-2Me-cAMP for 2 hours. As shown in Figure 5.5, the Epac specific analogue increased Id2 levels to a similar extent as 8-CPT-cAMP. The effect was concentration dependent. We found that Rap-1 was activated following treatment of J774.2 with 8-CPT-cAMP, 8-CPT-2Me-cAMP and forskolin (L. Gronning's personal communication). These results suggest that activation of EPAC mediates the induction of Id2 protein level in response to an increase in intracellular cAMP concentration.

5.3.5 Effect of cAMP requires PI3 Kinase.

Existing evidence shows that the Epac-dependent pathway can activate ERK or PI3 kinase signalling cascade in a cell-specific and differentiation stage-specific manner. The mitogenic effect of cAMP in thyroid cells is mediated through PI3-kinase/PKB (Cass, Summers et al. 1999; Ciullo, Diez-Roux et al. 2001) and PKB is phosphorylated in EPAC-expressing cells (Mei, Qiao et al. 2002) while B-Raf expression is required for Erk activation by Rap-1 (Fujita, Meguro et al. 2002). To identify the signalling cascade mediating the effect of cAMP on Id2 protein level in our system, J774.2 cells were pretreated with 100nM wortmannin, 30 μM LY29002 or 30 μM PD98059 prior to treatment with 8-CPT-cAMP. We found that the cAMP effects on Id2 expression required PI3-kinase but not the Erk cascade. As shown in Figure 5.6, we observed inhibition with both wortmannin and LY29002, two structurally dissimilar PI3 kinase specific inhibitors.

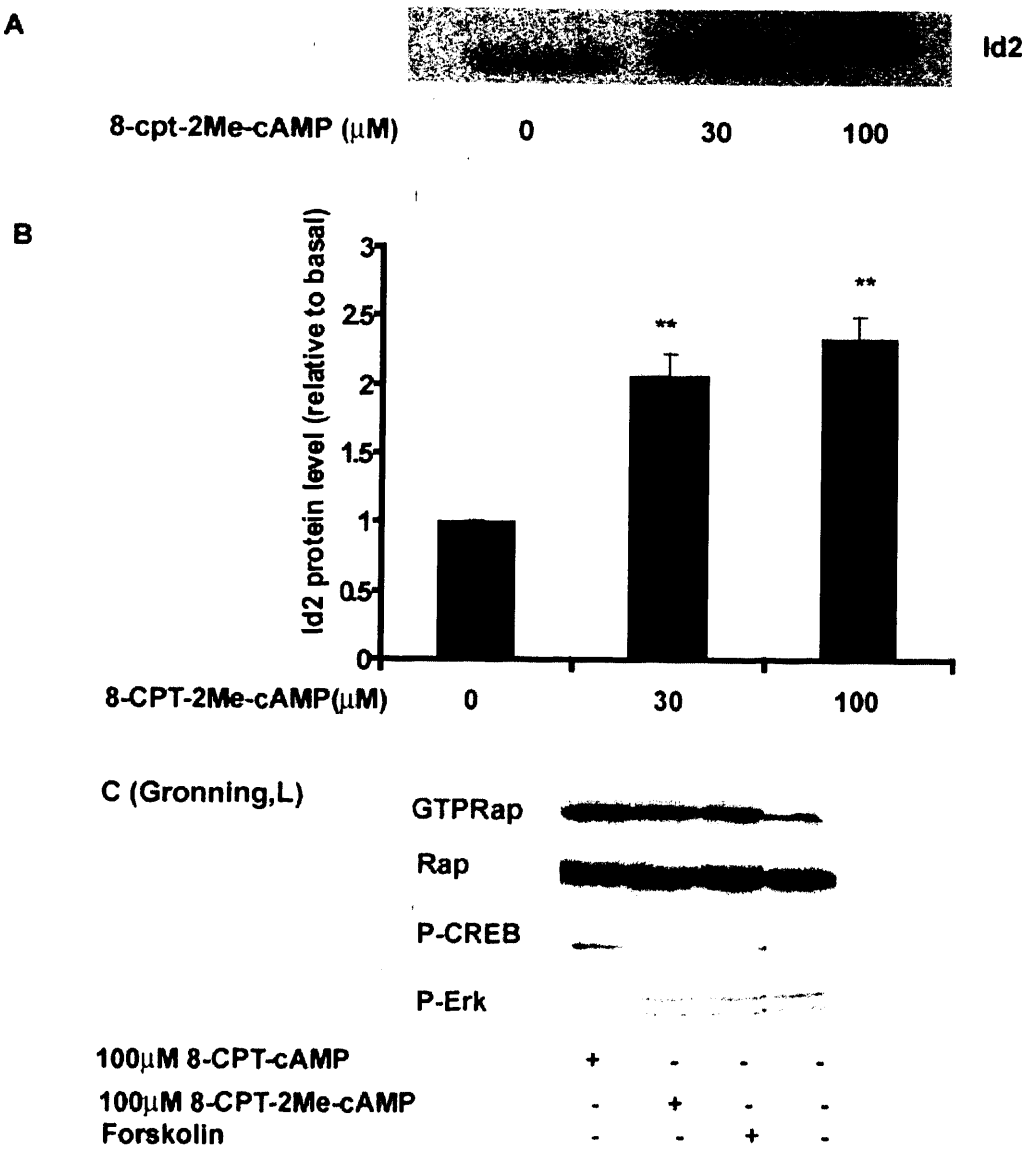


Figure 5.5 Epac-specific analogue of cAMP, the 8-CPT-2Me-cAMP mimics the effect of 8-CPT-cAMP and Rap-1 is activated by 8-CPT-cAMP in J774.2 macrophages

A: J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours cells were treated with 30 or 100 μM of 8-CPT-2Me-cAMP. Id2 was immunoprecipitated after 2 hours and relative Id2 protein level was determined by Western blotting. B: Quantitation of three individual experiments (mean +/- SEM). Statistical analysis was performed using student's paired t test. Significant differences are indicated where ** indicates where $p<0.05$. C: RAP-1 activation by 8-CPT-cAMP and Epac specific analogue (Gronning, L. personal communication).

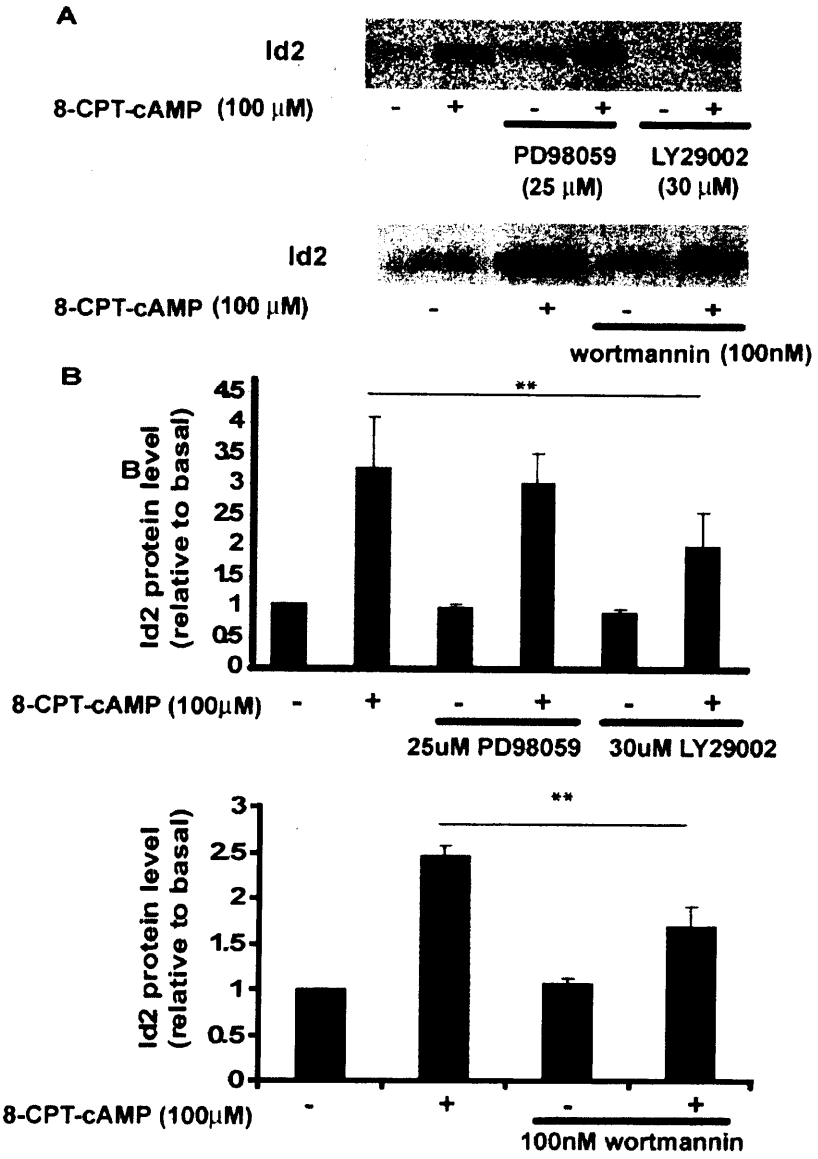


Figure 5.6 Effect of 8-CPT-cAMP requires PI3-kinase

A: J774.2 macrophages were incubated overnight in serum-free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours, cells were treated with 100nM of wortmannin, 30 μM or LY29002 or 25 μM of PD98059 for 30 minutes before the addition of 100 μM of 8-CPT-cAMP. Id2 was immunoprecipitated after 2 hours and relative Id2 protein level was determined by Western blotting. B: Quantitation of three individual experiments (mean ± SEM). Statistical analysis was performed using student's paired t test. Significant differences are indicated where ** indicates where $p<0.05$.

5.3.6 8-CPT-cAMP increase PKB phosphorylation in J774.2 cells

Having identified PI3-kinase as an important mediator, we examined the effect of cAMP on PKB and ERK activity following 8-cpt-cAMP treatment. PKB activity was determined by immunoblotting with an antibody that specifically recognises Ser473, a growth factor-regulated phosphorylation site required for PKB activation. We found that PKB phosphorylation increased with 8-CPT-cAMP treatment. Maximum response was reached at 100 μM of 8-CPT-cAMP. The response decreased when cells were treated with 200 μM of 8-CPT-cAMP. At 100 μM 8-CPT-cAMP, the phosphorylation was induced by 30 minutes and was sustained through to 2 hours as shown in Figure 5.7. We found that the cAMP effect on PKB phosphorylation was PKA-independent and could be replicated by an Epac-specific analogue (L. Gronning's personal communication). Our observation agrees with result published by Mei et al. Briefly, Epac and PKA mediates opposing effect of cAMP on PKB regulation. Activation of Epac leads to PKB activation, while stimulation of PKA inhibits PKB. The divergent mechanism provides a potential mechanism for cell type-specific effects of cAMP. The net outcome of cAMP treatment would depend on dynamic abundance and intracellular distribution of Epac and PKA (Mei, Qiao et al. 2002).

We also found divergent regulation of ERK by cAMP. In J774.2 cells, ERK phosphorylation increased with 50 μM of 8-CPT-cAMP then decreased with 100 μM 8-cpt-cAMP treatment. At 100 μM, the decrease continued up to the 2 hours time point as shown in Figure 5.7. Such concentration-dependent effects of cAMP on magnitude and duration of Erk phosphorylation is not uncommon. In Schwann cells, low concentration of cAMP promotes short and intense Erk activation while high concentration of cAMP promotes sustained but low activation (Mutoh, Li et al. 1998). It would be of some interest to carry out a more detailed analysis of very early time course at 2 and 5 minutes to assess Erk phosphorylation dynamic in our system, but such experiment is outside the scope of the present study.

In sum, our observations show that PKB phosphorylation responds to cAMP through a similar pathway and in a similar time course and dose response to the cAMP effect on Id2. This observation combined with the inhibitor studies suggest that cAMP regulates Id2 protein level through PI3 kinase and PKB.

5.3.7 Insulin increases Id2 protein level in J774.2 macrophages and in insulin sensitive tissues via a PI3-kinase dependent pathway.

Next, we investigated whether insulin affects Id2 expression in J774.2 macrophages. Cells were starved overnight in serum free media. After 18 hours, 100nM or1 μM insulin was

added to the cells. We found that insulin increased Id2 protein level considerably in J774.2 cells (Figure 5.8 A). As shown in Figure 5.8 B, the effect is PI3 kinase dependent. In view of the fact that insulin signalling is ubiquitous in most cell types, there is a possibility that insulin could affect Id2 expression in other insulin sensitive cells. We investigated the effect of insulin on L6 myocytes, HepG2 cells and differentiated 3T3-L1 adipocytes. We found that similar treatment in these cells generated similar response as shown in Figure 5.8 A. The discovery that Id2 is upregulated by insulin in these tissues opens up a new avenue for future research. Insulin inhibits many metabolic genes such as PEPCK in these tissues but the effect does not always correlate well with decrease in expression of a stimulator of transcription. A dominant negative regulator of transcription such as Id2 could mediate the inhibitory effect of insulin on metabolism-related genes by sequestering other bHLH transcription factors.

5.3.8 Insulin and 8-CPT-cAMP increase GSK3 phosphorylation

Having identified the PI3-kinase–PKB cascade as an important contributor to the upregulation of Id2 protein expression by cAMP and insulin, we considered downstream effectors of PKB which may mediate control on Id2 level. PKB is known to phosphorylate many of proteins with the consensus R*X*R*XX*(S/T) site. To date, more than 50 proteins summarised in the table 6.1 have been identified as putative target for PKB (Hanada, Feng et al. 2004).

Table 5.1 Target of PKB

Function	Name
Metabolism	Glycogen Synthase Kinase 3 α and β
	Phosphodiesterase 3B
	cardiac-specific isoform of 6-phosphofructo-2-kinase (6-PF2-K)
	mammalian target of rapamycin (mTOR)
	products of the tumour suppressor genes TSC1 and 2
	Protein tyrosine phosphatase (PTP1B)
Regulation of apoptosis	BAD (Bcl-2/Bcl-X antagonist) / Caspase9
	Apoptosis signal-regulating kinase 1 (ASK1) MKKK
	mixed lineage kinase 3 (MLK3) / SEK1/MKKK4
Transcription factor	FKHR/FoxO1, FoxO2, FKHRL1/FoxO3 and AFX/FoxO4 IKK
	cyclic AMP (cAMP)-response element binding protein

	orphan nuclear receptor Nurr77
	Androgen receptor (AR)/ Yes-associated protein (YAP)
Cell cycle regulator	p21 / p27/kip1 / MDM2 (controversial)
	ARK5 (AMPK)
	Myt1
Other targets	eNOS
	Raf
	p47 phox NADPH oxidase
	Grb2-associated binding protein 2 (Gab2)
	EDG-1 (endothelial differentiation, sphingolipid G-protein-coupled receptor, 1)

the substrates mentioned above, GSK3 is the most interesting candidate. It has been implicated in similar cellular functions as PKB and Id2, namely the control of cell cycle. Moreover, GSK3 regulates the level and activity of many transcription factors such as CREB, HIF, C/EBP, c-myc and NF-kB by phosphorylating and inactivating or targetting them for degradation. PKB inhibits GSK3 kinase activity by phosphorylating N terminal serine residues. On GSK3-alpha, serine residue 9 is the target while serine residue 21 is the target on GSK3 alpha (Cross, Alessi et al. 1994; Cross, Alessi et al. 1995). Phosphorylation of these residues is inhibitory as it converts N-terminal of GSK3 into a pseudosubstrate for GSK3 domain that binds to priming phosphate on GSK3 targets. This mechanism prevents target binding and blocking substrate access to the active site on GSK3 without affecting GSK3 activity (Frame, Cohen et al. 2001). Filippa et al show that PKB activated by cAMP translocated to the plasma membrane and phosphorylated GSK3 in a similar manner to those activated by insulin, further suggesting that GSK3 could be an important link in the control of Id2 protein expression.

To investigate the involvement of GSK3, we first assessed whether GSK3 phosphorylation is affected by cAMP and insulin in J774.2 cells. Macrophages were serum starved overnight. After 18 hours cells were treated with 100 μM of 8-CPT-cAMP or 1 μM of insulin. PhosphoGSK3 was detected with phosphospecific antibody by Western blotting. Basal GSK3 phosphorylation is relatively low in these cells. Treatment with insulin and 8-CPT-cAMP increased GSK3 phosphorylation significantly. The effects were clear after 1 hour of stimulation with 8-CPT-cAMP and insulin as shown in Figure 5.9. As shown in Figure 5.10, the effect of 8-CPT-cAMP was replicated by the Epac-specific analogue 8-CPT-2Me-cAMP. Pre-treatment of J774.2 cells with 100nM wortmannin abolished the increase in GSK3

phosphorylation following 8-CPT-cAMP and 8-CPT-2Me-cAMP treatment, but PD98059 had no effect (Figure 5.10). The observation confirms that activation of PKB by cAMP and insulin in J774.2 macrophages lead to increased GSK3 phosphorylation via a pathway requiring Epac and PI3 kinase activity.

5.3.9 GSK3 specific inhibitors increase Id2 protein level in J774.2 macrophages.

Recently, it has been shown that inactivation of GSK3 leads to increased stability and accumulation of several factors such as alphaNAC, RAP1GAP, c-MYC, β-catenin, NF-kappa B1 (Foltz, Santiago et al. 2002; van Noort, Meeldijk et al. 2002; Gregory, Qi et al. 2003; Quelo, Akhouayri et al. 2004; Tsygankova, Feshchenko et al. 2004). In order to confirm that GSK3 inhibition directly leads to the increase in Id2 protein level, serum starved J774.2 macrophages were treated with 3 μM or 30 μM of GSK3 specific inhibitors SB216763 or SB415286 for 4 hours. Both inhibitors are structurally distinct maleimides and have been proven to be activators of glycogen synthesis and β-catenin expression (Coghlan, Culbert et al. 2000). The concentration used in this experiment falls within the range previously used in hepatocytes (Lochhead, Coghlan et al. 2001). The protein level of Id2 increased with increasing concentration of GSK3 inhibitors as shown in Figure 5.11. We conclude that that inhibition of GSK3 plays an important role in the increase of Id2 protein level. Since both cAMP and insulin activate PKB in this system, inactivation of GSK3 could be the common pathway in which cAMP and insulin increase Id2 protein level in J774.2 macrophages.

5.3.10 cAMP effect is additive with glucose

Previously we have shown that high glucose increases Id2 protein level. In order to assess whether glucose acts synergistically with cAMP, confluent J774.2 macrophages were serum starved overnight in RPMI 1640 media before being transferred to RPMI media containing either 5mM or 20mM glucose. After 18 hours, cells were treated with 100 μM of 8-CPT-cAMP. We found that glucose and cAMP acted additively to increase Id2 protein level in J774.2 macrophages as shown in Figure 5.12 A. Next we assessed whether similarly additive effects occurred with the combination of insulin and 8-CPT-cAMP. As shown in Figure 5.12 B, the effect was not additive. Id2 protein level reached its maximum with 8-CPT-cAMP treatment and insulin treatment did not enhance the level further. This observation suggests that insulin and cAMP work through the same pathway mediated by GSK3 while hexosamine pathway mediates the glucose effect separately, leading to significantly additive effect between glucose and 8-CPT-cAMP.

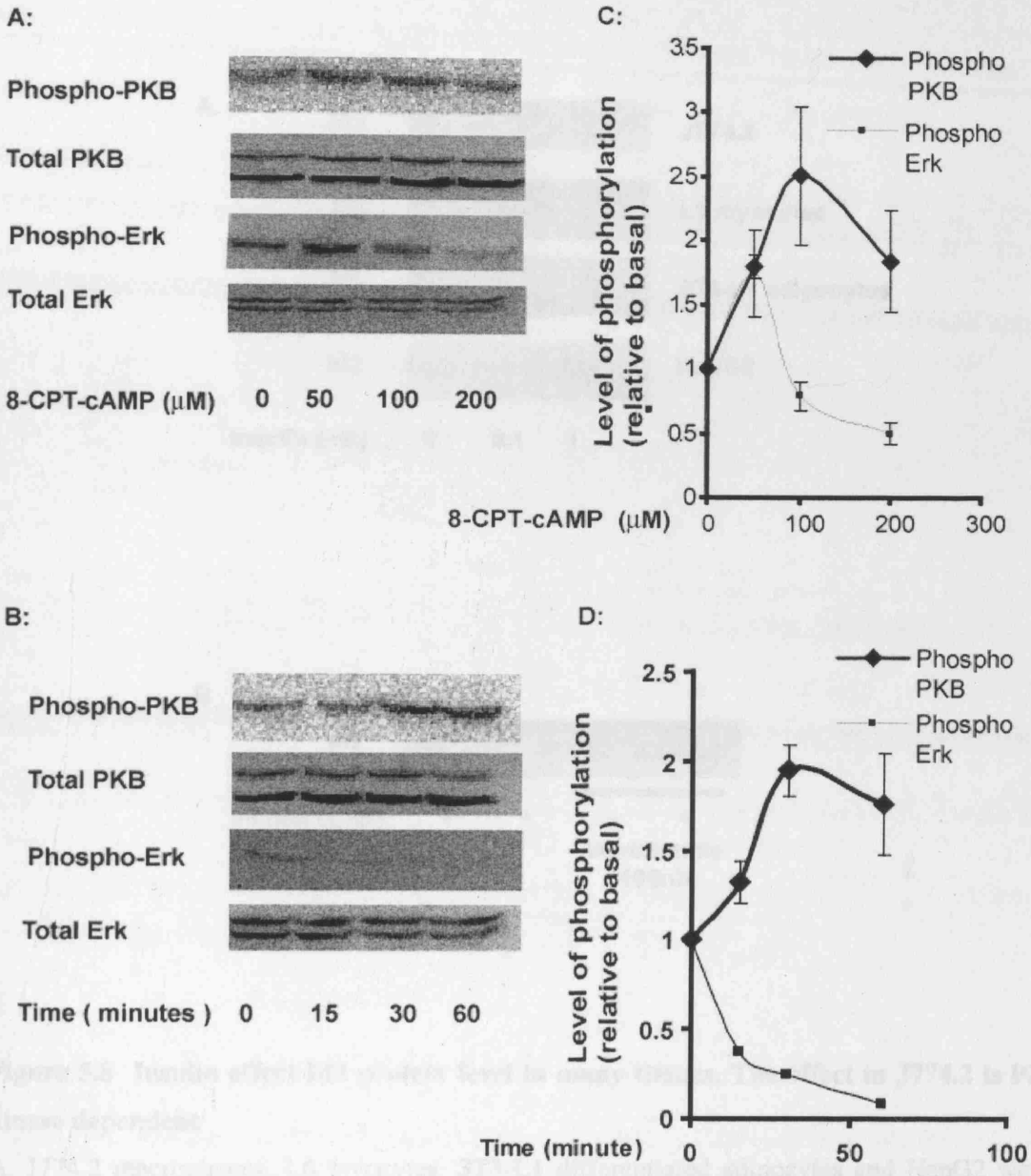


Figure 5.7 Effect of 8-CPT-cAMP on PKB and Erk phosphorylation in J774.2 cells

J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours, cells were treated with 100 μM of 8-CPT-cAMP for 15, 30 or 60 minutes (A) or cells were treated with 50, 100 or 200 μM of 8-CPT-cAMP for 30 minutes (B) Relative amount of phosphorylated PKB and Erk were detected by Western blotting. (C,D) Graphical representation of (A) and (B) in successive order. Data represent mean ± SEM of two independent experiments.

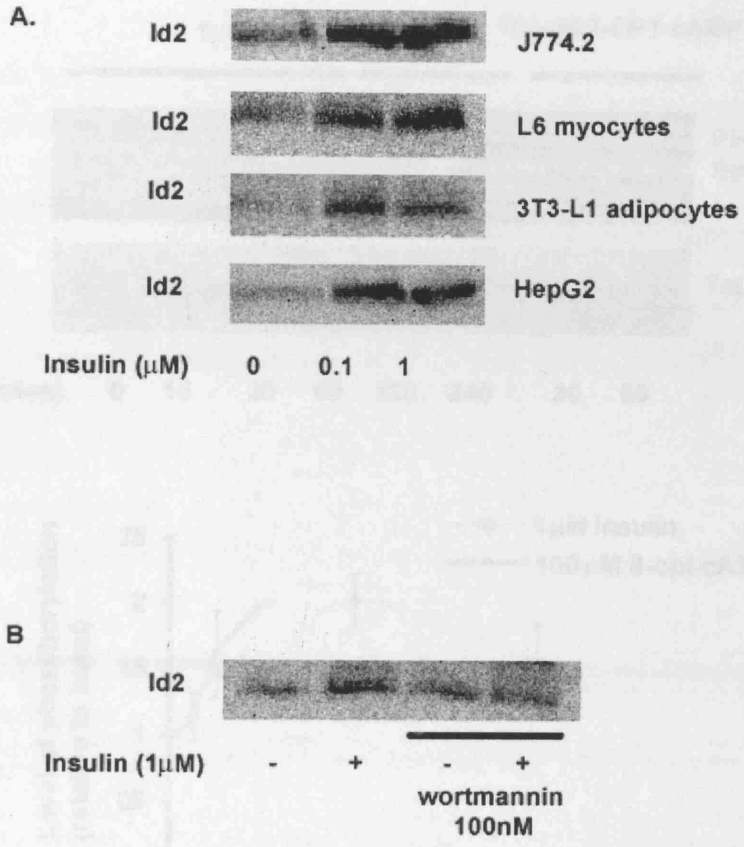


Figure 5.8 Insulin effect Id2 protein level in many tissues. The effect in J774.2 is PI3 kinase dependent

A: J774.2 macrophages, L6 myocytes, 3T3-L1 differentiated adipocytes and HepG2 were treated with 100nM or 1μM of insulin for 4 hours. Id2 was immunoprecipitated and relative Id2 protein level was determined by Western blotting. Representative blots from three separate experiments are shown. B:J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours, cells were treated with 100nM of wortmannin for 30 minutes before the addition of 1μM of insulin. Representative blots from three separate experiments are shown.

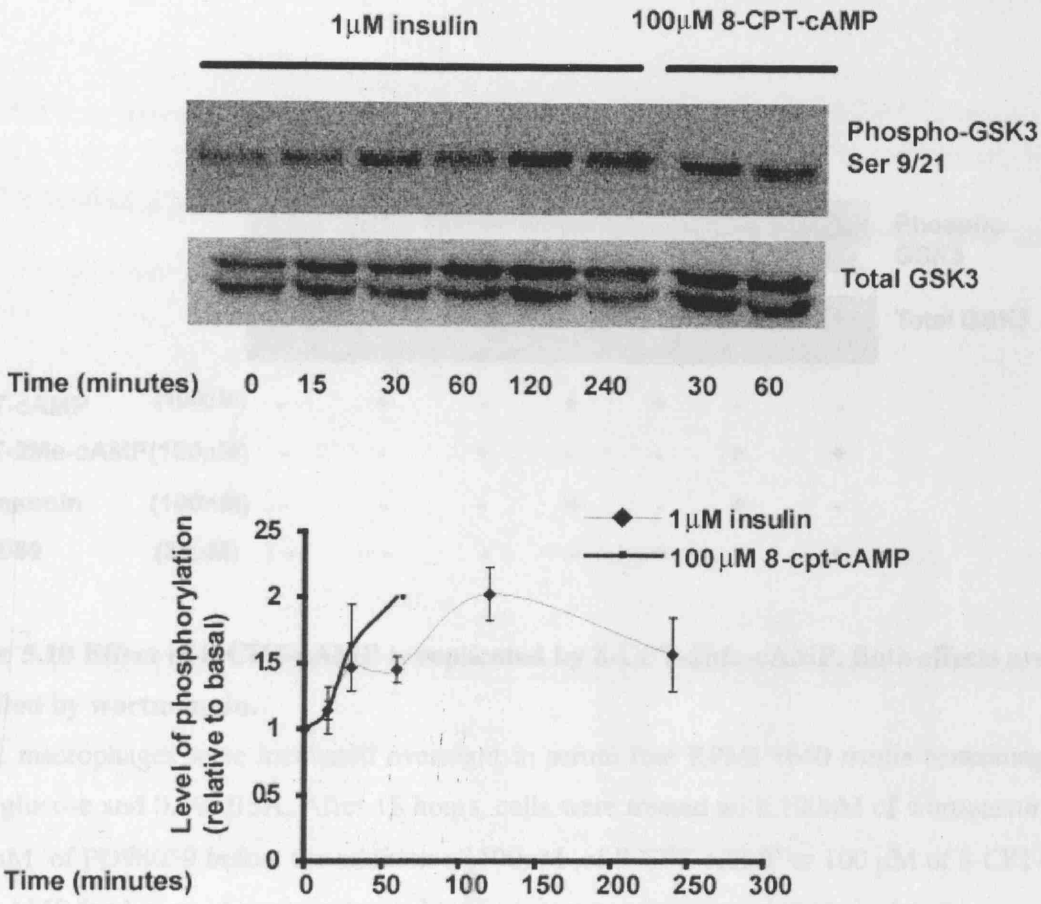


Figure 5.9 Insulin and 8-CPT-cAMP increases GSK phosphorylation on Ser 9/21 in J774.2 macrophages.

A: J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours, cells were treated with 100 μM of 8-CPT-cAMP or 1μM of insulin for the designated time. Relative amount of phosphorylated GSK3 was detected by Western blotting. B: Quantitation of two separate experiments.

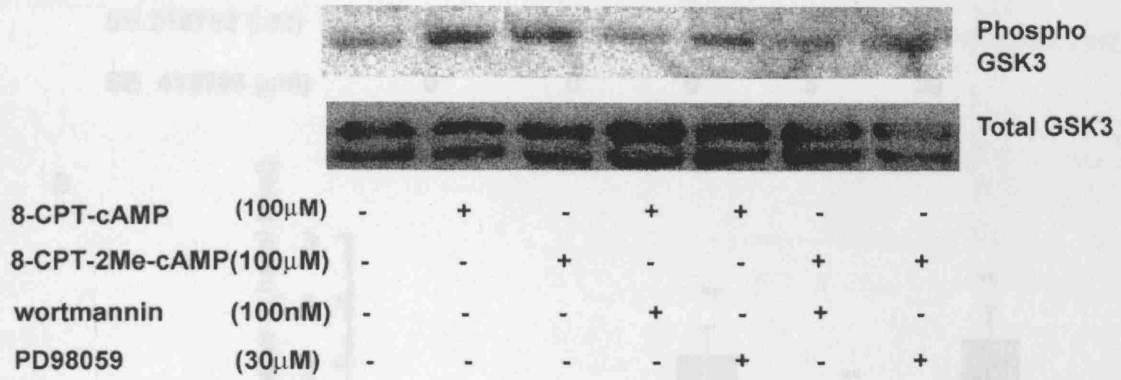


Figure 5.10 Effect of 8-CPT-cAMP is replicated by 8-CPT-2Me-cAMP. Both effects are inhibited by wortmannin.

J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours, cells were treated with 100nM of wortmannin or 25μM of PD98059 before the addition of 100μM of 8-CPT-cAMP or 100 μM of 8-CPT-2Me-cAMP for 1 hour. A representative blot from two separate experiments is shown.

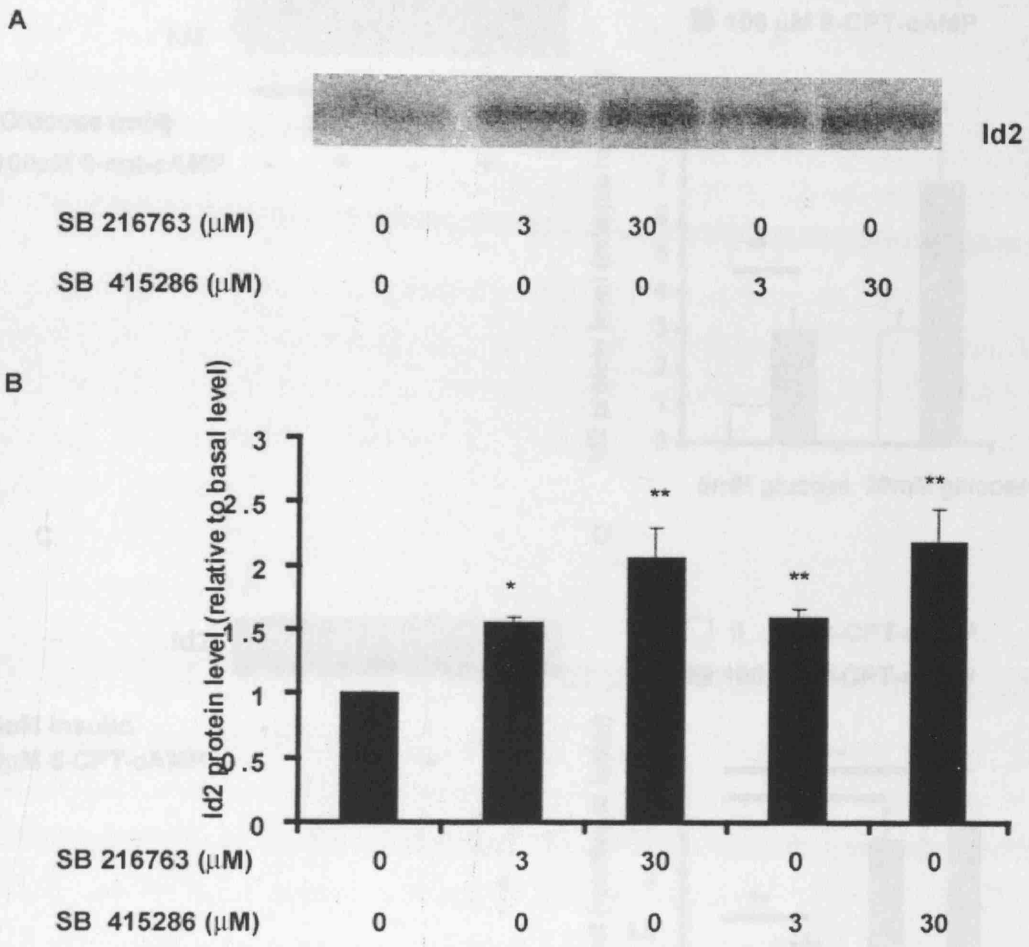


Figure 5.11 GSK3 inhibitor mimics the effect of 8-CPT-cAMP and insulin on protein level of Id2 in J774.2 macrophages

A:J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours cells were treated with 3 or 30 μM of GSK3 specific inhibitors SB 415286 or SB 216763. Id2 was immunoprecipitated after 2 hours and relative Id2 protein level was determined by Western blotting. B: Quantitation of three individual experiments (mean +/- SEM). Statistical analysis was performed using student's paired t test. Significant differences are indicated where ** indicates where $p<0.05$.

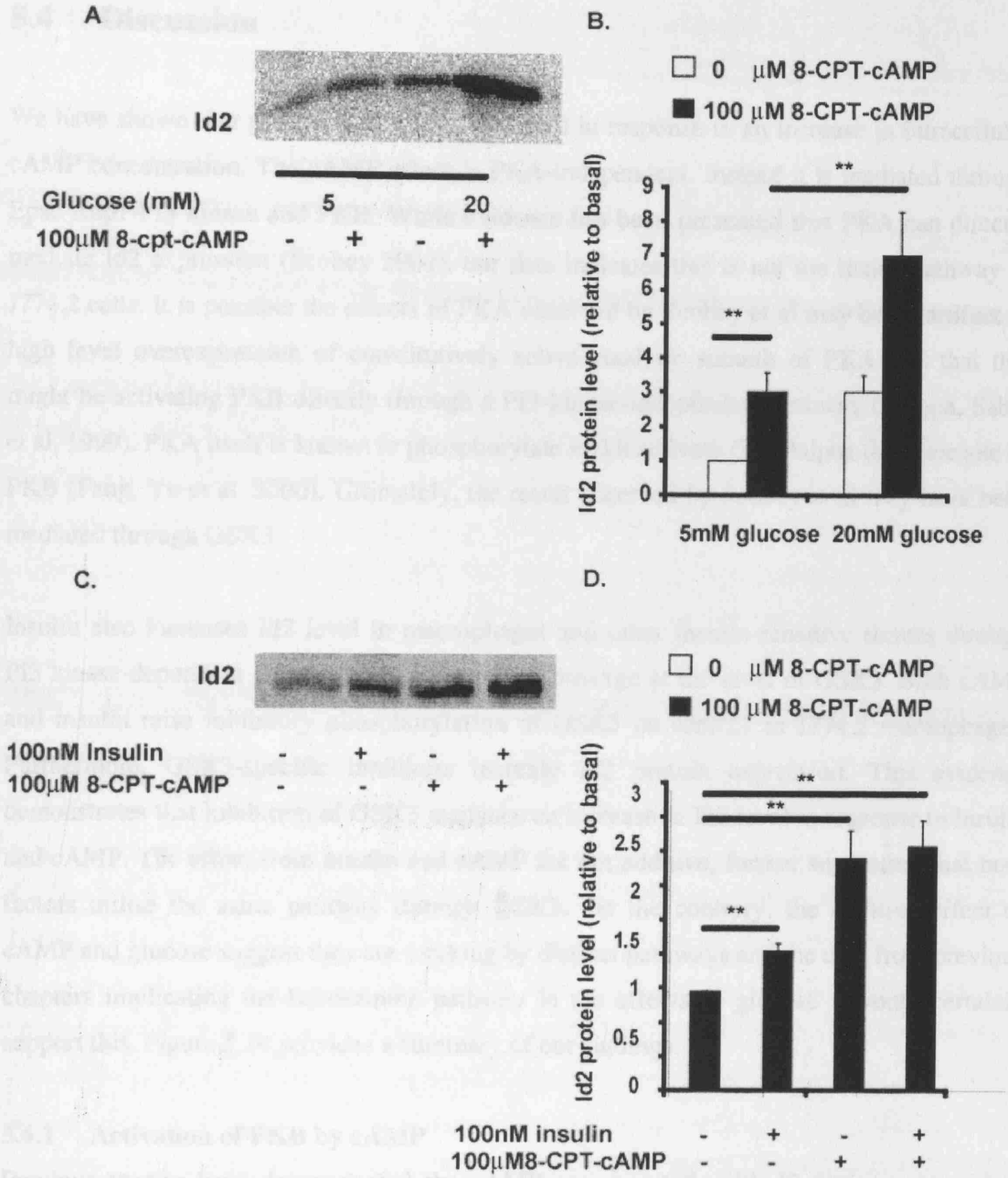


Figure 5.12 The cAMP effect is additive with glucose but not additive with insulin

A: J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM or 20 mM glucose and 0.2% BSA. After 18 hours cells were treated with 100 μM of 8-CPT-cAMP for 2 hours. Id2 was immunoprecipitated and relative Id2 protein level was determined by Western blotting. C: J774.2 macrophages were incubated overnight in serum free RPMI 1640 media containing 5mM glucose and 0.2% BSA. After 18 hours, cells were treated with 100nM of insulin, 100μM of 8-CPT-cAMP or a combination of both. Id2 was immunoprecipitated after 2 hours and relative Id2 protein level was determined by Western blotting. B/D: Quantitation of three individual experiments (mean +/- SEM). Statistical analysis was performed using student's paired t test. Significant differences are indicated where ** indicates where $p<0.05$.

5.4 Discussion

We have shown that protein level of Id2 is raised in response to an increase in intracellular cAMP concentration. The cAMP effect is PKA-independent. Instead it is mediated through Epac-Rap1-PI3 kinase and PKB. While evidence has been presented that PKA can directly mediate Id2 expression (Scobey 2004), our data indicates this is not the major pathway in J774.2 cells. It is possible the effects of PKA observed by Scobey et al may be an artifact of high level overexpression of constitutively active catalytic subunit of PKA and that this might be activating PKB directly through a PI3-kinase-independent pathway (Filippa, Sable et al. 1999). PKA itself is known to phosphorylate and inactivate GSK3alpha the same site as PKB (Fang, Yu et al. 2000). Ultimately, the result observed by Scobey et al may have been mediated through GSK3.

Insulin also increases Id2 level in macrophages and other insulin-sensitive tissues through PI3 kinase-dependent pathway. Both pathways converge at the level of GSK3. Both cAMP and insulin raise inhibitory phosphorylation of GSK3 on ser9/21 in J774.2 macrophages. Furthermore, GSK3-specific inhibitors increase Id2 protein expression. This evidence demonstrates that inhibition of GSK3 mediates an increase in Id2 level in response to insulin and cAMP. The effect from insulin and cAMP are not additive, further suggesting that both factors utilise the same pathway through GSK3. On the contrary, the additive effect of cAMP and glucose suggest they are working by distinct pathways and the data from previous chapters implicating the hexosamine pathway in the effects of glucose would certainly support this. Figure 5.14 provides a summary of our findings.

5.4.1 Activation of PKB by cAMP

Previous studies have demonstrated the cAMP can crosstalk with PI 3-kinase-dependent pathways at the level of PKB. Evidence for cAMP activation of PKB through PKA-independent mechanism first came from a study involving cAMP-stimulated cell proliferation in thyroid cells (Coulonval, Vandeput et al. 2000). Inhibition of PKA does not block the proliferative effect of cAMP in these cells; neither can constitutively active C subunit of PKA replicate the proliferative effect of cAMP. In 1998, Cass et al demonstrated that TSH, forskolin and a cAMP analogue all promote phosphorylation as well as activation of p70s6k and PKB. PKB phosphorylation was not affected by H89. Rather, it was blocked by wortmannin and LY294002. Both PI3-kinase specific inhibitors also block TSH-stimulated thyroid cell proliferation.

Subsequently, observations in thyroid cell have been replicated in many cell types. In twenty-day old rat Sertoli cell culture, an increase in PKB phosphorylation has been observed following treatment with FSH and cAMP analogue. The increase is abolished by PI3-kinase inhibitors. Wortmannin and LY294002 significantly inhibit FSH-stimulated transferrin secretion and lactate production by inhibiting PKB-regulated processes of glucose transport and lactate dehydrogenase activation (Meroni, Riera et al. 2002; Meroni, Riera et al. 2004).

PKA-independent activation of PKB plays an important role in granulosa cells, where growth and differentiation depends on the interplay between LH and FSH, both raising intracellular cAMP by activating adenylate cyclase. In small follicles, FSH stimulates granula cell proliferation by upregulating cyclin D2. As follicles mature, FSH stimulates expression of LH receptor. Subsequent LH surge then curtails proliferation and stimulates terminal differentiation, reducing the level of cyclin D2 and LH receptor while increasing expression of genes for luteinisation. In differentiated granulosa cell expressing LH receptor, FSH is found to stimulate PKB phosphorylation through PI3-kinase dependent mechanism (Gonzalez-Robayna, Falender et al. 2000). In luteinised granulosa cells, generation of cAMP by LH leads to Epac-mediated progesterone secretion(Chin and Abayasekara 2004).

The mechanism plays an important role in cellular functions other than proliferation and differentiation. PKB activation by cAMP contributes to regulation of cellular architecture. In Dictyostelium, cAMP causes translocation of PKB to the leading edge of the membrane and its subsequent activation (Gao, Knecht et al. 2004). In hepatocytes, activation of PKB is involved with the cAMP-mediated increase in bile acid transport and translocation of Ntcp, a serine/threonine phosphoprotein involved in bile acid transport(Webster, Blanch et al. 2002).

Overall, the effect of cAMP on PKB phosphorylation is cell type-specific and correlates well with the mitogenic effects of cAMP (Cass, Summers et al. 1999). In cells in which cAMP is mitogenic, cAMP stimulates proliferation as well as membrane ruffling. On the other hand, PKB phosphorylation is not observed in cells where cAMP inhibits proliferation such as NIH3T3 fibroblasts and smooth muscle cells. The protein mediating PKA-independent effects of cAMP remained a mystery until the discovery of Epac. Epac is an exchange factor for the small GTPase Rap-1 and Rap-2. Epac1 is a ubiquitously expressed protein while Epac2 has limited expression in brain, liver and adrenal gland. Epac contains a DEP (dishevelled, Egl-10, Pleckstrin) homology domain that targets them to the membrane, thus positioning them in proximity to other membrane-localised enzyme such as PI3-kinase, PDK-1 and PKB. The mechanism by which cAMP regulates Epac function has been

elucidated by biochemical study and x-ray crystallography of Epac2. In the holo form of Epac, the cAMP binding domain masks GEF domain and inhibits its catalytic exchange activity. Upon cAMP binding, conformational change allows the release of GEF domain and activation of its catalytic activity. (Rehmann, Rueppel et al. 2003; Rehmann, Schwede et al. 2003)

A study by Mei et al. demonstrates that Epac is the main mediator of cAMP effect on PKB phosphorylation. In HEK293 cells stably transfected with Epac, cAMP causes three-fold induction of PKB phosphorylation and kinase activity in comparison with parental non-transfected cells expressing negligible amount of Epac(Mei, Qiao et al. 2002). Likewise, Rap-1 activation is ultimately necessary for PKB activation by cAMP in rat thyroid cells. The ability of TSH to stimulate PKB phosphorylation is enhanced by the expression of activated Rap1A and markedly repressed in cells expressing a putative dominant negative Rap1A(Tsygankova, Saavedra et al. 2001). Our observation in J774.2 cells agrees with these previous findings. We found that cAMP increases Id2 levels through a PI3-kinase-dependent pathway, which is mediated through Epac via a PKA-independent mechanism. The observed increase in Rap-1 activation and PKB phosphorylation is consistent with this.

The exact mechanism linking Epac to PI3-kinase activation is unknown at present. One possibility involves the similarity between effector binding regions of Ras and Rap1. Ras can form a complex with and activate p110α and p110γ subunit of PI3 kinase (Rodriguez-Viciana, Warne et al. 1994; Pacold, Suire et al. 2000). Because of the similarity, Rap may bind Ras effectors such as the p110 subunit of PI3 kinase. Alternatively Rap may facilitate the movement of PI3 kinase to biological membrane or act on phosphatases regulating PI3 kinase and PKB. On the other hand, another cAMP-sensitive guanine nucleotide exchanger specific for Ras has been discovered. This protein is named (CNrasGEF) (Pham, Cheglakov et al. 2000). It contains cyclic nucleotide rasGEF, which contains CDC25, Ras exchange motif (REM), Ras-association (RA), PDZ and cNMP (cAMP/cGMP) binding (cNMP-BD) domains, two PY motifs and a carboxy-terminal SxV sequence. CNrasGEF can also activate the small GTPase Rap1 in cells, but this activation is constitutive and independent of cAMP. Therefore, it is a possibility that the Epac-specific analogue may activate CNrasGEF *in vivo* and that the PKA independent effect leading to PI 3-kinase activation and PKB phosphorylation has been mediated by CNrasGEF activation of Ras.

5.4.2 Inhibition of Erk by cAMP

Results presented here show that 8-cpt cAMP causes a decrease in Erk phosphorylation. PKA-mediated inhibition of Raf is a likely mechanism. Studies in several cell types such as smooth muscle cells, fibroblasts, NIH3T3, HEK293 (Burgering, Pronk et al. 1993; Graves, Bornfeldt et al. 1993; Wu, Dent et al. 1993; Schmitt and Stork 2001) have shown that activation of protein kinase A can inhibit growth factor-induced Erk signalling through a target downstream of Ras and upstream of Raf-1. PKA directly inhibits Raf-1 activity by phosphorylating Serine 43, 259 and 621 residues (Mischak, Seitz et al. 1996). Phosphorylation of Serine 259 is sufficient for inhibition of full length Raf-1 by PKA (Dhillon, Pollock et al. 2002), and dephosphorylation of Serine 259 is essential for the interaction between Raf-1 and Ras as well as MEK (Dhillon, Pollock et al. 2002). An alternative mechanism depends on PKA-dependent activation of Rap-1. In fibroblasts, PKA activates Rap-1 indirectly through Rap1 guanine nucleotide exchange factor C3G, Cbl scaffolding protein and tyrosine kinase Src. PKA first activates Src by directly phosphorylating Serine 17. Src phosphorylates tyrosine residue on Cbl. This phosphorylated residue acts as docking site for SH2 domain of Crk-L and C3G. The complexes are recruited to the plasma membrane.(Schmitt and Stork 2002). Subsequently GTP-loaded Rap-1 binds to and sequesters Raf-1 away from Ras, causing an inhibition of the Erk cascade (Schmitt and Stork 2001).

Alternatively a direct inhibition of Raf by PKB may have occurred. Evidence shows that PKB is a potent inhibitor of Raf-1. In C2C12 differentiated myotubes, activated PKB inhibit phosphorylation on Ser 338, which is required for Raf activation. The inhibition is differentiation stage-specific and such interaction does not exist in myoblasts (Rommel, Clarke et al. 1999). Raf-1 also contains a highly conserved PKB motif R*X*R*XX*(S/T). In MCF-7 breast cancer cell line and vascular smooth muscle cells, PKB inhibits Raf-1 by phosphorylating inhibitory residue Ser 259 within the PKB phosphorylation motif (RQRSTS^{259}). Subsequent reduction in Raf-1 activity is caused by binding of Ser 259 phosphorylated Raf-1 to the inhibitory 14-3-3 protein(Zimmermann and Moelling 1999; Reusch, Zimmermann et al. 2001). In all cases, Raf-1 inhibition by PKB leads to reduction in Erk phosphorylation. Our results contradict the observation made by Enserink et al in which Epac-specific analogue of cAMP fails to inhibit Erk phosphorylation in NIH-3T3 cells. Nonetheless, cAMP inhibits proliferation in NIH3T3 cells (Burgering, Pronk et al. 1993) and it might not activate PI3 kinase/PKB in that particular system.

5.4.3 Intracellular cAMP raising agents and Id2

The studies with adrenaline, leptin and LPA confirm that agents capable of raising intracellular concentration of cAMP results in the increase of Id2 protein level, albeit to a much lesser extent than that caused by the cell permeable analogue (8-CPT-cAMP). Bernard et al have also previously noted a similarly poor response to adrenaline in J774.2 cells. Several possible reasons exist for this discrepancy. One possibility is that J774.2 cells may express only low levels of receptors for these ligands and subsequently only generates small amounts of cAMP. While J774.2 have been reported to contain β-adrenergic receptors (Chambaut-Guerin and Thomopoulos 1987) and leptin receptor (O'Rourke 2001), we have not verified the expression levels in our particular clonal cell line. The other possibility is that the endogenously produced cAMP is continually being degraded by phosphodiesterase (PDE's) so limiting the magnitude of the effect. Our results support this as the PDE inhibitor IBMX increased both basal and LPA-induced expression of Id2. IBMX was used instead of PDE subtype-specific inhibitor because the exact identity of the PDE in J774.2 cells is unclear. O' Rourke et al found that these cells do not express PDE3B (O'Rourke 2001). PDE4 is a likely candidate as many isotypes of PDE4 have been detected in J774A.1 clonal cell line (Lin 2002), U937 monocyte/macrophage cell line and human monocyte/macrophage (Shepherd, Baillie et al. 2004). PDE4-specific inhibitor such as rolipram or cilomilast might exert a similar effect to IBMX in our system.

The finding that cAMP-raising agents can induce Id2 expression could be physiologically relevant in the development of atherosclerosis, particularly in the case of LPA. LPA is a naturally occurring phospholipid ligand for G protein coupled receptor. In physiological system, LPA is produced by platelets (Eichholtz, Jalink et al. 1993), cancer cells (Shen, Belinson et al. 1998) and adipose tissues(Pages, Rey et al. 1999). Activated platelets secrete phospholipids that provide a pool of lysophospholipids that are substrates of lysophospholipase D-mediated LPA production in plasma (Eichholtz, Jalink et al. 1993). In the plasma, LPA is mainly found as a component of minimally oxidized LDL (Maschberger, Bauer et al. 2000). LPA is closely linked to atherosclerotic development. In atherosclerotic plaques that were removed by surgery from human carotid arteries and aorta LPA was found to have accumulated in the intima with highest concentration around lipid rich core (Siess and Tigyi 2004) and increased vascular concentration of LPA has been correlated with the development of atherosclerotic lesions, mainly through its ability to increase platelet aggregation (Siess, Zangl et al. 1999; Maschberger, Bauer et al. 2000).

LPA affects many aspects of macrophages functions. J774.1A macrophages express LPA1/Edg2 receptor, while human monocytes express both LPA1 and LPA2. LPA1 receptor

is downregulated as human monocytes differentiate into human macrophages (Lee, Liao et al. 2002; Duong, Bared et al. 2004). Studies have shown that LPA increases TNF alpha production in murine macrophages(Lee, Liao et al. 2002). LPA also increases the concentration of Ca^{2+}, a second messenger of cellular activation, in human monocytic Monomac 6 cells (Fueller, Wang de et al. 2003). LPA has been recognised as a lipid with growth factor-like property. LPA stimulates proliferation of fibroblasts, (Kumagai, Morii et al. 1993; Jalink, Hordijk et al. 1994) whereas in terminally-differentiated peritoneal macrophages with no proliferative capacity, LPA promotes cell survival through anti-apoptotic mechanisms with equal potency to serum (Koh, Lieberthal et al. 1998). LPA activation of PI3-kinase is essential for mitogenic and anti-apoptotic effect of LPA (Kumagai, Morii et al. 1993; Koh, Lieberthal et al. 1998). Evidence shows that G-protein-coupled receptor can activate p110γ directly (Stoyanov, Volinia et al. 1995; Lopez-Ilasaca, Crespo et al. 1997) but this mechanism has not been confirmed as the mechanism underlying the LPA effect in peritoneal macrophages.

LPA affects cAMP formation in a cell-specific manner. In PC12 cells and fibroblast, LPA inhibits cAMP formation through Gαi activation (Carr, Grassie et al. 1994; Tigyi, Fischer et al. 1996). In airway smooth muscle cells, LPA elicits an opposing response. In these cells, LPA is responsible for the increase in intracellular cAMP level in response to serum (Nogami, Whittle et al. 1995). The βγ subunits released from LPA-activated receptors are primarily responsible for the activation of adenylate cyclase. Gq receptor-activated by LPA also triggers phosphoinositide turnover, which leads to PKC activation and induction of adenylate cyclase. LPA also inhibits activity of phosphodiesterase enzyme (Mamillapalli, Haimovitz et al. 1998), leading to accummulation of intracellular cAMP. Since LPA is present ubiquitously in the body at physiologically relevant concentrations, diverse effects of LPA are probably mediated by multiple subtypes of G protein-coupled LPA receptors with distinct signalling properties and tissue distribution. The effect of LPA, therefore, must be investigated on a cell type-specific basis. A study by Gronning et al confirmed that LPA raised cAMP levels in J774.2 macrophages especially in combination with IBMX. The exact mechanism through which LPA increase intracellular cAMP has not been studied in detail in J774.2 cells. However extensive study in RAW 264.7 macrophages and human monocyte-derived macrophages have shown that LPA activates adenylate cyclase through the activation of atypical PKC (Lin, Chang et al. 1999).

To date, the end effector of mitogenic and anti-apoptotic effect of LPA has not been identified. Our observation that LPA increases Id2 protein levels is highly significant. Mitogenic and apoptotic effects of Id2 have been well documented in many cell types.

Mitogenic effect of LPA requires PI3-kinase activation; while upregulation of Id2 by cAMP in our system also requires PI3-kinase. These parallel observations suggest that Id2 contributes to the mitogenic effects of LPA and LPA could be responsible for induction of Id2 expression by serum. In order to verify this hypothesis, it would be necessary to check if removal of Id2 expression, possibly through RNAi, inhibits the mitogenic and anti-apoptotic effect of LPA. At present, it remains unclear whether induction of Id2 by LPA results in anti-apoptotic or mitogenic effects in J774.2 cells. Unlike, terminally differentiated peritoneal macrophages, J774.2 are a leukaemia-derived cell line and they maintain proliferative capacity. Future experiments must resolve this matter and similar studies should be carried out with human monocyte-derived tissue macrophages to establish whether this observation has important implication in the development of atherosclerosis.

There is a possibility that LPA may exert its effects through pathways other than the upregulation of intracellular cAMP level since LPA can stimulate p110γ through βγ subunit of G protein. Nonetheless, co-addition of IBMX, a non-selective PDE inhibitor, with LPA caused a larger increase in protein level of Id2 than observed with either alone. The synergistic effect suggests that the LPA effects on Id2 expression arise through its ability to raise intracellular cAMP. To confirm that LPA exert its effects through cAMP, it would be necessary to apply an inhibitor of adenylate cyclase or an inhibitor of atypical PKC isoforms with LPA. Possible PKC-specific inhibitors are bis-indolylmaleimide or Go6983, which inhibits all isoform except PKCmu while SQ22536 could be used as an adenylate cyclase inhibitor.

Leptin is another hormone of interest. Macrophages are gaining a reputation as a significant target for leptin. Leptin receptors are abundant on the surface of primary macrophages(Gainsford, Willson et al. 1996) and the Long form of leptin receptor or OB-R_L with signalling capability is expressed on J774.2 macrophages. Tyrosine phosphorylation of leptin receptor and JAK-2 as well as an increase in PI3-kinase lipid kinase activity are detected in these cells following leptin administration (O'Rourke 2001). Leptin also activated Erk phosphorylation in a biphasic manner with initial decrease and subsequent activation after 30 minutes (O'Rourke 2002). How leptin generates intracellular cAMP in J774.2 cells remains a mystery. However, a lack of PDE3B expression in J774.2 macrophages (O'Rourke 2001) may account for an increase in cAMP following leptin treatment as PDE3B is responsible for the reduction in cAMP level in islet cells (Zhao, Bornfeldt et al. 1998).

Leptin affects many macrophages functions. It increases the expression of interferon-gamma-inducible-protein (IP-10) in monocytes, leading to the recruitment of activated T-cells to lesion area. It increases the expression of lipoprotein lipase in J774.2 macrophages via a PKC and oxidative stress-dependent mechanism(Maingrette 2003). Leptin also enhances phagocytosis (Gainsford, Willson et al. 1996) and production of proinflammatory cytokines including m-CSF from macrophages (Loffreda 1998). Finally, leptin increases the activity of Acyl CoA Cholesterol O-acyltransferase (ACAT) enzyme in J774.2 in high glucose condition, causing accelerated accumulation of cholesterol ester and accelerated foam cell formation (O'Rourke 2002). The observation that Id2 levels are altered by leptin suggests that in addition to STAT3, Id2 could be another mediator of leptin's effect on gene expression. Id2 may exert positive effects on gene expression by binding to and sequestering another negative regulator of transcription. The physiological significance of Id2 expression in response to leptin remains to be analysed. Similar to LPA, leptin might affect Id2 expression through direct activation of PI3 kinase. It is important to verify whether the cAMP-generating effect is important in this case by utilising adenylate cyclase inhibitors.

5.4.4 Location of intracellular cAMP generation may underline deviation in magnitude of response to the cell permeable analogue 8-CPT-cAMP and other intracellular cAMP raising agents.

The observed response discrepancy between each intracellular cAMP raising ligands in our system could be accounted for by differences in location, duration, and amplitude and extent of cAMP generation. Location of cAMP generation is an important determinant of the outcome of cAMP generation. Compartmentalisation of cAMP allows spatially different pools of effector proteins to be activated. In living cells, spatial and temporal control of cAMP signalling is achieved by anchorage of every component of the cascade from ligand receptor, adenylate cyclase, Phosphodiesterase enzyme, protein kinases and phosphatases to a specific subcellular compartment (Tasken and Aandahl 2004). G protein-coupled receptor and adenylate cyclase with transmembrane domains are usually localised together in membrane caveolae or lipid rafts (Schwencke, Yamamoto et al. 1999) (Zaccolo, Magalhaes et al. 2002). Cyclic AMP generated from the plasma membrane only diffuses up to approximately 1μm. Therefore it has been suggested that soluble adenylate cyclases which are attached to mitochondria, nuclear envelope and microtubules are responsible for the activation of cAMP effector near the nucleus (Zippin, Chen et al. 2003). Free diffusion of intracellular cAMP is limited by phosphodiesterases and their importance to signalling process is confirmed by studies in PDE-deficient mice(Jin, Richard et al. 1999). Subcellular location of PDE 4 is precisely controlled through targeting by scaffolding proteins such as AKAP, arrestin, RACK1, src or phosphatidic acid in case of PDE4A1. The examples are

summarised in table 5.2. Binding of PDE to these proteins occurs through UCR2 domain or the N-terminal region and is under the control of phosphorylation by PKA and Erk. Isoenzymes of PDE contain different sequences, hence each binds to different sets of scaffolding proteins, resulting in a diverse array of subcellular targetting. In fact, different isozymes of PDE in the same cell type can control separate pools of cAMP, resulting in two separate cellular responses from the same effector. For example, in mesangial cells, PDE3 controls cAMP pools that suppress proliferation while PDE4 direct the pool that controls ROM generation, although both processes require PKA (Chini, Grande et al. 1997).

Anchorage proteins concentrate effector proteins together in an area where cAMP is generated and is thus at high levels. Cyclic-AMP effector such as PKA and Epac are also localised in a very specific manner. AKAP proteins target PKA to a specific pool of cAMP (Michel and Scott 2002), specific substrates and subcellular compartment. Epac is under a similarly rigorous control (Qiao, Mei et al. 2002). It is mainly localized to the nuclear membrane and mitochondria during interphase. In metaphase, Epac is localized to the mitotic spindle in metaphase. Cyclic AMP plays an important part in Epac targetting. High concentrations of cAMP target Epac to the nuclear membrane while low concentration target Epac to microtubules. The significance of Epac targetting has not been unraveled but it is a subject under intense study.

While 8-cpt-cAMP cell permeable analogue generates uniformly distributed cAMP responses, various hormones utilized in this study generate a more localized pool of cAMP with different magnitude. LPA may generate cAMP in a cellular region that is more favorable to Id2 induction than adrenaline. Location is particularly important for Epac-mediated signalling as Epac (Km 10μM) has much lower affinity for cAMP than PKA (Km 100nM). This implies that Epac-mediated signalling cascade is activated when high concentration of cAMP is generated in an area with less abundant phosphodiesterase and in close proximity to downstream effector. Magnitude of intracellular cAMP generation could be a factor determining the ultimate signalling outcome. It would be interesting to analyse differences in cAMP accumulation as a result of LPA, Leptin, Adrenaline and IBMX treatment in J774.2 cells. Recently, a valuable cAMP florescence indicator system based on cAMP-dependent conformational change of Epac has been used to monitor spatiotemporal dynamic of cAMP signalling in living cells (DiPilato, Cheng et al. 2004). Such a system would shed useful information on the differences generated by each hormone in our system.

Table 5.2 PDE anchorage

PDE isozyme	scaffold protein	target
PDE3s(Degerman, Belfrage et al. 1997)	trnasmembrane domain	endoplasmic reticulum
PDE4D5(Yarwood, Steele et al. 1999)	RACK-1	
PDE4D3(Jin, Bushnik et al. 1998)	myomegalin	golgi/centrosome
PDE4D3 (Dodge, Khouangsathiene et al. 2001)	muscle AKAP	perimuclear region
PDE4D / PDE4A(Beard, O'Connell et al. 1999; Beard, Huston et al. 2002)	bind SH3 Src kinase	
PDE4 catalytic domain (MacKenzie and Houslay 2000)	bind to and phosphorylated by Erk	
PDE4A1(Baillie, Huston et al. 2002)	bind PI through TAPAS	membrane
PDE4 (Baillie, Sood et al. 2003)	arrestin	

5.4.5 Possible mechanisms underlying GSK3 regulation of Id2 protein level

Induction of Id2 by two structurally distinct GSK3 inhibitors, together with increases in GSK3 phosphorylation in response to 8-CPT-cAMP and insulin suggests that inhibition of GSK3 mediates the effects of insulin and cAMP on Id2 expression. Phosphorylation by GSK3 affects either stability or activity of transcription factors. Firstly, phosphorylation by GSK3 targets several proteins such as c-Myc (Sears, Nuckolls et al. 2000) and Rap1Gap (Tsygankova, Feshchenko et al. 2004) to ubiquitin-mediated proteasomal degradation. Part of the cellular GSK3 pool is present in a multiple protein complex involving axin, APC and β-catenin. Phosphorylation of β-catenin by GSK3 creates a recognition site for binding of the F-box protein, bTrCP, which acts as the recognition subunit for the E3 ubiquitin ligase (Aberle, Bauer et al. 1997; Hart, Concordet et al. 1999). The complex targets β-catenin for proteolysis. Thus while GSK3 is active, *β*-catenin is continually being degraded. Wnt signalling disrupts the interaction between axin and GSK3, thereby preventing β-catenin degradation (li 1999). Secondly, direct phosphorylation by GSK3 inhibits activity of SREBP-1 (Kim, Song et al. 2004), c/EBP *β* (Ross 1999; Piwien-Pilipuk, Van Mater et al. 2001), c-Jun, NFAT and HSF1. The inhibition is reversed once GSK3 is inhibited through PKB-mediated phosphorylation. GSK3 also forms an inhibitory complex with GATA factor. When GSK3 is phosphorylated, the complex is dissembled. GATA then enters the nucleus and activates GATA site and various promoters (Morisco, Seta et al. 2001).

Id2 is degraded through ubiquitination-mediated proteasomal degradation (Bounpheng, Dimas et al. 1999; Fajerman, Schwartz et al. 2004)}. The existence of putative GSK3 phosphorylation sites on Id2 suggest that phosphorylation by GSK3 may contribute to its proteasomal targetting. Inhibition of GSK3 by PKB and dephosphorylation of Id2 in turn would lead to its accummulation.

As described in chapter 3, the putative GSK3 phosphorylation site on Id2 (SDHSLGISRSKTPVDDPMS) contains several serine/threonine residues in the correct SxxxS consensus (Fiol, Wang et al. 1990). Since GSK3 is a proline-directed kinase (Jho, Lomvardas et al. 1999), the presence of proline residue on the +1 position from the threonine residue in Id2 further suggests that this protein could be a novel target of GSK3. Substrate for GSK3 phosphorylation can be segregated into primed substrate and non-primed substrate. The primed substrate requires priming phosphorylation by another kinase on the n+4 serine residue. This phosphorylation brings GSK3 into close proximity to its substrate by binding to arginine 96 on GSK3. A good example of a primed substrate is c-Myc. GSK3 phosphorylates c-Myc on threonine 58 given that another kinase phosphorylates priming site on ser 62 (Sears, Nuckolls et al. 2000). On β-catenin (LDSGIHSGATTTAPS[45]LS), Casein Kinase Iα primes serine 45 residue before GSK3 phosphorylates threonine 41, serine 37 and serine 33 (Liu, Li et al. 2002). In contrast, the non-primed substrates such as axin (RTPGRQSPGPGHRSPDS) (Frame, Cohen et al. 2001) and cyclin E (SPLPSGLLTPP) (Welcker, Singer et al. 2003) do not require priming phosphorylation. Instead of the association between arginine 96 on GSK3 and priming phosphorylated serine on the substrates, axin bind to leucine 126 residue on GSK3 (Frame, Cohen et al. 2001). Priming phosphorylation might not be necessary for Id2 since D (aspartic acid) can act as a substitute for phosphorylated serine/threonine. Consequently, GSK3 could be brought into close proximity with Id2 through the interaction between aspartic acid residue and arginine 96 residue without prior phosphorylation by another kinase. On the other hand, Id2 might be a non-primed substrate and the presence of proline residue next to threonine could be sufficient to direct GSK3 to phosphorylate the protein as in the case of cyclin E.

Alternatively, GSK3 inactivation may stabilise or activate other transcription factors that bind to Id2 promoter, resulting in transcriptional activation of Id2. This is highly likely as previous study in Sertoli cells have shown that cAMP affects activity of Id2 promoter. Scobey et al have shown that the -122 to -82 region on human Id2 promotor is essential for cAMP response in sertoli cells but they fail to identify a transcriptional activator. Previously Rockman et al (Rockman, Currie et al. 2001) have shown that β-catenin binds to the -95

residue and activates human Id2 promoter in colon carcinoma cells. Since β-catenin is stabilised by GSK3 inactivation, there is a possibility that accumulation of β-catenin may underline increase Id2 expression. Evidence remains conflicting as to whether β-catenin can be stabilised by insulin-dependent inactivation of GSK3. Ding et al show that stabilisation only occur with Wnt, a glycoprotein but Satyamooythy show that insulin can stabilise β-catenin in melanoma cells. (Ding, Chen et al. 2000; Satyamoorthy, Li et al. 2001)The effect could be cell-specific because of differences in scaffolding complex and further research in macrophages is required.

Analysis of the mouse Id2 promoter shown in Figure 5.13 reveals a cluster of E boxes, two of which contain high affinity Myc binding sites, which are fully conserved in human Id2 promoter. Mouse promoter also contains binding sites for GATA proteins, β-catenin, Sp-1 and C/EBP β, all of which are known to be activated through GSK3 inactivation. Further analysis using real-time PCR quantification, bandshift, luciferase assay involving Id2 promoter and overexpression of the factors mentioned above would clarify the main contributors to the effect of cAMP on Id2 expression.

5.4.6 Functional implications

The increase in Id2 protein level by cAMP in macrophages may have many functional implications. Many processes in macrophages ranging from H_2O_2 production, bacteriocidal activity and phagocytosis are inhibited by cAMP via Epac-mediated signalling (Aronoff, Canetti et al. 2005). Id2 may participate in the inhibition by suppressing transcription.

Classically, insulin and cAMP are perceived as two opposing signals. However, recent findings challenge the antagonistic nature of both hormones. In skeletal muscle, adrenaline actually potentiates insulin-stimulated phosphorylation of PKB and of PKB activity through Epac activation. The effect is replicated by Epac-specific analogue of cAMP although this analogue does not activate glycogen phosphorylase (Brennesvik, Ktori et al. 2005). The findings suggest that outcome of cAMP signalling actually depends on the cell-specific balance of PKA and Epac activity. When PKA is the main mediator, the outcomes are antagonistic to those of insulin. However, when Epac activity is predominant, cAMP may activate similar pathway to insulin and the effects could be synergistic.

Our results provide another system where cAMP and insulin are not antagonistic. Our observations also have implications for the control of cell proliferation. Effects of cAMP on cell proliferation are quite varied. In many cells such as Rat1 and NIH3T3 (Burgering, Pronk et al. 1993; Graves, Bornfeldt et al. 1993; Wu, Dent et al. 1993), cAMP inhibits cell growth

and acts antagonistically towards growth factors. In other cells such as PC12, Swiss 3T3, thyrocytes and most importantly macrophages (Frodin, Peraldi et al. 1994; Withers, Bloom et al. 1995; Misra, Akabani et al. 2002; Misra and Pizzo 2002), cAMP can promote cell proliferation by promoting the G1/S phase transition in cell cycle. In these cells, cAMP effects on proliferation are similar to the effects of insulin and growth hormones such as IGF. Mitogenic effect of cAMP correlates well with the activation of the PI3kinase/PKB pathway. In cells in which cAMP is mitogenic, cAMP stimulates PKB phosphorylation and membrane ruffling through a PKA-independent pathway (Cass, Summers et al. 1999; Ciullo, Diez-Roux et al. 2001). Our observation suggests that Id2 could be the effector for mitogenic effects of cAMP as well as IGF. Our signalling analyses, which show that cAMP acts through Epac—Rap—PI3 kinase—PKB to stimulate Id2, a known promotor of proliferation, fits perfectly with existing literature. Id2 is a known inducer of G1/S phase progression, the same effect activated by cAMP. It would be important to carry out cell proliferation assays in the future to clarify functional significance of Id2 upregulation in response to insulin and cAMP raising agents in macrophages.

Our findings also have implications for the mechanism of accelerated atherosclerotic development in diabetes. Evidence supporting a proatherogenic role of Id2 came from a study in ApoE/Id2 double knock out mice. Lesions in these mice are 40% smaller in size than those in single ApoE knockout mice. Our study shows that hyperglycaemia enhances the normal Id2 induction in response to cAMP. In relative terms, priming of macrophages in high glucose increases the cAMP induction of Id2 protein from 2-fold up to 6-fold. The observation suggests that hyperglycaemia contributes to overinduction of Id2 in response to normal inducers. Too much Id2 protein may cause pathological effects intracellularly by interfering with normal protein-protein and protein-DNA interaction. Uncontrolled interaction of Id2 with other proteins as a result of hyperglycaemia could contribute to accelerated atherosclerotic development in diabetes. At present, the mechanism underlying proatherogenic property of Id2 is unclear. Reductions in HSL expression mentioned in chapter 5 could be a contributing factor but potentially Id2 may interact with a wide array of other proteins, leading to a proatherogenic outcome.

The increases in Id2 in response to insulin in hepatocytes, adipocytes and muscle also have important functional implications in metabolism. Insulin inhibits the expression of many gluconeogenic genes such as Phosphoenol pyruvate carboxykinase (PEPCK) in hepatocytes at the level of transcription. The inhibition requires inhibition of GSK3 by PI3-kinase and PKB, but the exact regulatory molecule has not been identified (Lochhead, Coghlan et al. 2001). PEPCK promoter also contains SRE sites and E box sites, Id2 potentially could

interfere with the binding of helix-loop-helix factors at these sites. Promoter and co-transfection studies would clarify this hypothesis.

Figure 5.13 Analysis of mouse Id2 promotor E-box position obtained from (Neuman 1995). Initiation codon position obtained from (Mantani 1998). Other potential transcription factor binding sites are predicted with MetInspector program. (Quandt, Frech et al. 1995)

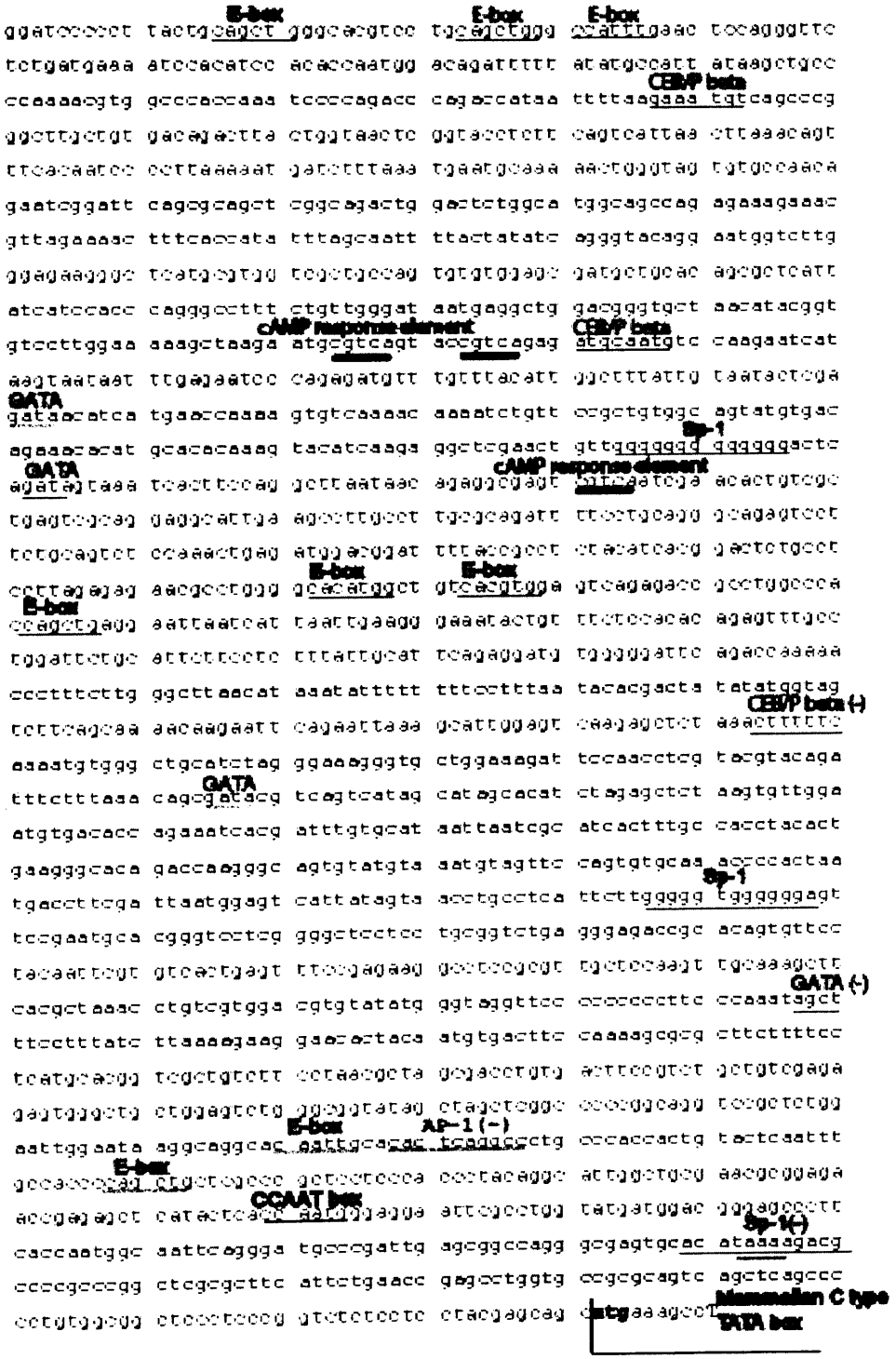
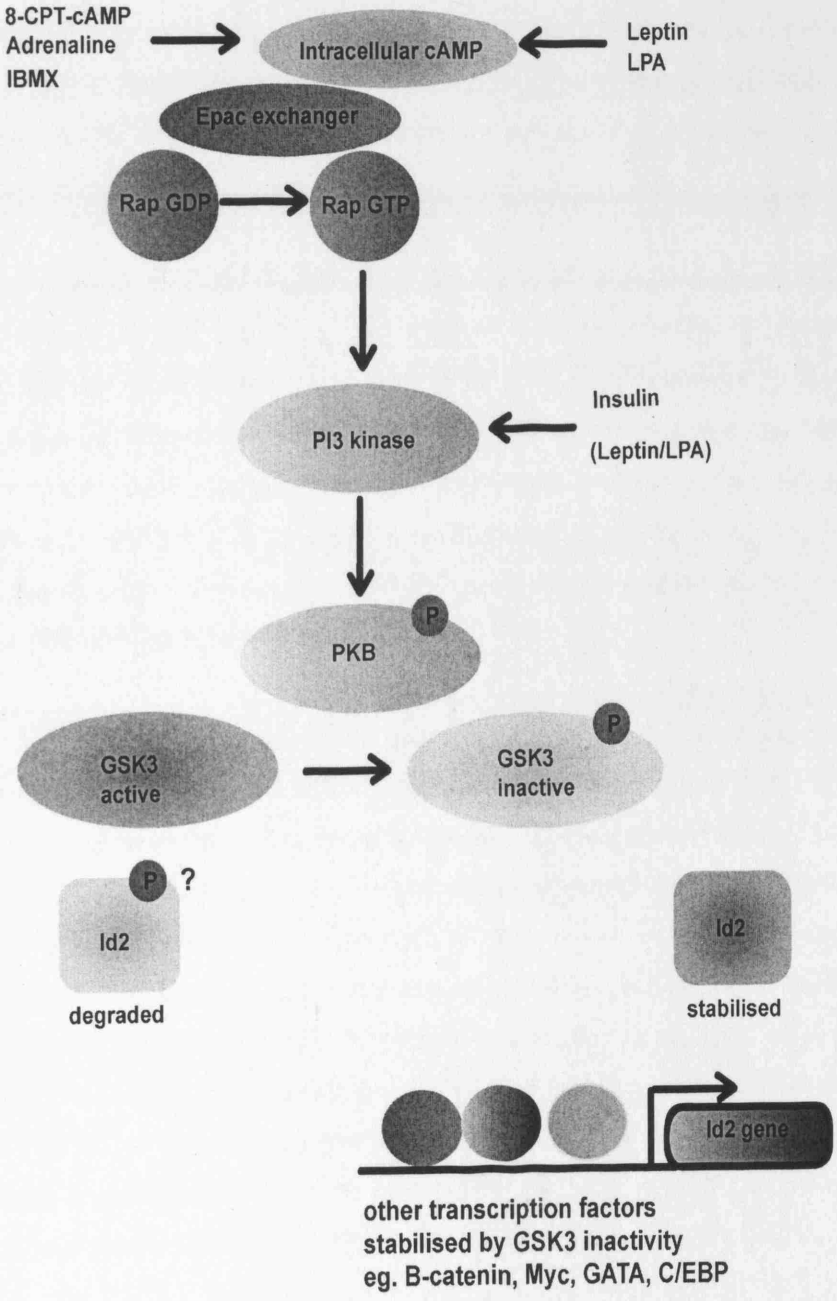


Figure 5.14 Schematic representation of the increase in Id2 protein level by cAMP and Insulin

An increase in intracellular cAMP concentration activates Epac, which converts GDP-bound Rap into the active GTP bound form. Activated Rap signals to PI3-kinase, causing the generation of membrane-bound phospholipid. PKB is recruited to the membrane through its PH domain. At the membrane it is phosphorylated and activated by PDK1. Activated PKB phosphorylates GSK3 on ser9/21, inhibiting GSK3 activity. Many transcription factors, possibly including Id2, are phosphorylated and targetted for proteasomal degradation by GSK3 phosphorylation. Inactivation of GSK3 leads to the accummulation of Id2 and other transcription factors that activate the Id2 promoter.



5.5 Conclusion

In conclusion, this chapter provides evidence that cAMP and insulin use a convergent pathway to regulate Id2 expression with the cAMP pathway employing the novel cAMP effector EPAC. The convergent pathway uses requires PI3-kinase, PKB and GSK3. This provides the first evidence for the existence of this pathway.

Chapter 6 Conclusion and future directions

Accelerated atherosclerosis development continues to be one of the main causes of mortality in diabetic patients. Previously, work by O'Rourke et al suggested that changes in levels and activity of HSL in macrophages as a result of hyperglycaemia, hyperinsulinaemia and hyperleptinaemia could cause increased cholesterol deposit in foam cells. This thesis attempts to identify the mechanisms linking these factors to changes in HSL levels. The research carried out identified changes in SREBP and Id2 levels and showed that these changes have the potential to affect HSL promoter activity. Extensive analysis has been carried out to elucidate the mechanism linking changes in glucose levels to changes in amount of Id2 protein in macrophages. Additionally, further analysis regarding the effects of hormones on Id2 levels in macrophages have been carried in later part of the thesis. Many avenues of further research have been generated from the work and several possibilities are mentioned here.

In chapter 3, we have shown that the changes in SREBP-1 levels mimicked changes in HSL promoter activity very closely. Furthermore, SREBP-1 co-expression activated HSL promoter activity. To analyse the contribution of SREBP to HSL promoter regulation in macrophages, we should perform band shift assays and promoter deletion analysis. The same analyses should be carried out in primary human macrophages and human macrophage cell line. RNAi inhibition of SREBP-1 or the maturation machinery such as SCAP and Insig as well as other pharmacological inhibitors of SREBP-1 processing could be utilised to assess the contribution of SREBP-1 to HSL regulation.

In chapter 4, we demonstrated that Id2 levels increased in response to high glucose. The effect was mediated through hexosamine flux as confirmed by the increase in Id2 levels in response to glucosamine treatment, GFAT overexpression and azaserine treatment. Id2 itself was modified by o-linked glycosylation. To confirm this post-translational modification, His tagged Id2 could be expressed in insect cell such as Sf9, which contains the necessary machinery for o-linked glycosylation (Greis, Gibson et al. 1994). Id2 could be purified by affinity chromatography and the carbohydrate moiety analysed (Cheng, Cole et al. 2000). Briefly, the GlcNAc residue would be labelled with (^{3}H) Gal and the nature of glycosylation identified by the method of removal. Asparagine-linked N-glycan would be removed by PNGase while serine and threonine O-glycans would be released by dilute alkali-induced beta elimination (Roquemore, Chou et al. 1994). The product from beta elimination would

behave as disaccharides when fractionated using gel filtration chromatography. The attachment site could be identified by first digesting (^{3}H)-Gal labelled protein with trypsin and fractionating the tryptic fragments by reverse phase C_{18} chromatography. The mass of eluted peptide could be resolved with MALDI-TOF mass spectrometry (Cheng, Cole et al. 2000). The site of glycosylation could be identified by mutagenesis. It would be vital to analyse the functional consequences of this modification. Association between the modified or the unmodified forms of Id2 and other helix-loop-helix or helix-loop-helix leucine zipper containing factors should be analysed. We should look at the effects of glycosylation on localisation of Id2 since its location is known to be tightly regulated through nucleocytoplasmic shuttling (Kurooka and Yokota 2005) in a differentiation stage-specific manner (Tu, Baffa et al. 2003).

In chapter 4, we observed a decrease in SREB- mediated induction of HSL promoter when Id2 was co-expressed in macrophages. To further investigate the contribution of Id2 to changes in macrophage lipid metabolism, Id2 should be over expressed or knocked down using RNAi and various lipid parameters assessed in an animal model. So far we have carried out our analyses in mouse cell line, it would be interesting to verify the findings in human monocyte-macrophage cell line such as THP-1 and primary human macrophages.

In chapter 6, we observed an increase in Id2 levels in response to 8-CPT-cAMP, various intracellular cAMP raising agents and insulin. The effect was mediated through inhibitory phosphorylation of GSK3 as confirmed by the application of two structurally distinct GSK3 specific inhibitors. GSK3 controls levels of other proteins by phosphorylating and targeting them to ubiquitin-mediated proteasomal degradation. Phosphorylation by GSK3 targets several proteins such as c-Myc (Sears 2000) and Rap1Gap (Tsygankova, Feshchenko et al. 2004) and β-catenin (Aberle, Bauer et al. 1997; Hart, Concordet et al. 1999)for proteolysis. Id2 is degraded through ubiquitin-mediated proteasomal degradation (Bounpheng, Dimas et al. 1999; Fajerman, Schwartz et al. 2004). The presence of putative GSK3 phosphorylation sites, which is specific to Id2 and is conserved across many species, together with the increase in Id2 levels in response to GSK3 inhibitors suggests that Id2 could be a target of GSK3.

Future experiments should verify whether Id2 is a physiological target of GSK3. First, the test should be carried out *in vitro*. Id2 can be expressed in E.Coli using $pTYB_2$-based expression vectors, which yield recombinant proteins devoid of an associated fusion moiety. These can be incubated with recombinant GSK3 in an *in vitro* kinase assay. The maltose binding protein (MaBP) produced can be used as a negative control, whereas the

recombinant protein myelin basic protein (MyBP) can be used as positive control for the phosphorylation by GSK3. Exact location of phosphorylation sites could be mapped by point mutagenesis. When the exact site has been identified, phospho-specific antibodies could be raised and used to test for phosphorylation in vivo in response to various extracellular stimuli. The balance between phosphorylation and o-linked glycosylation at a particular site could be investigated. To investigate the physiological relevance of GSK3 phosphorylation, a knock-in homozygous mouse model in which the PKB phosphorylation sites on GSK3 alpha (serine 21) and GSK3 beta (serine 9) have been changed to alanine could be employed. These mice have been used to show that GSK3 beta (serine 9) phosphorylation is the major route by which insulin activates muscle glycogen synthase (McManus, Sakamoto et al. 2005). Contribution of GSK3 phosphorylation to Id2 stability should be investigated. Wild type and mutated Id2 could be expressed in CHO cells and cycloheximide study carried out to assess their half life. In the future it would be interesting to study target genes of Id2 and the physiological effects of an increase in Id2 expression in response to glucose and GSK3 inactivation in macrophages. An increase in Id2 levels does not necessary lead to a decrease in expression levels of target genes. In several cases, Id2 sequested another negative regulator of gene expression, allowing another positive regulators of transcription access to the target promoters. The complex nature of its physiological impact could be assessed by proteomic analysis. Id2 could be removed from certain tissues by RNAi or overexpressed using transfection before the changes in levels of protein expression from the system compared by proteomic analysis.

In addition to queries raised during the course of this research, further analysis of Id2 itself could be carried out. Phosphorylation sites on oncogenes such as Myc and β-catenin usually constitute mutation hot spots in cancer. Id2 itself is gaining a reputation as another oncogene. Mutation of its phosphorylation sites may contribute to cancerous development. It would be interesting to investigate the physiological effects of mutation by overexpressing mutated forms of Id2 in mice and to look for natural mutation in human population. Furthermore, the completion of Human Genome Project should facilitate searches for polymorphism in Id2 genetic sequences.

So far, we have not looked at the effects of changes in lipid profile on Id2 expression. The contribution of dyslipidaemia and hypercholesterolemia to atherosclerosis lesion progression might be mediated through Id2 since LDL and Id2 double knock out mice develop smaller atherosclerotic lesion in comparison to LDL knock out mice. Ox-LDL particles are known to affect the expression of several genes. Furthermore, LPA is one of its components. Since we have shown that LPA administration upregulates Id2 in J774.2 cells, there are high

possibilities of direct effects on Id2 expression from ox-LDL particles. We should look at the effects of oxLDL loading in macrophages and monocytic cell line as well as in primary macrophages and monocytes from humans. Because the progression of atherosclerotic lesion also involves the participation of leukocytes, smooth muscle cells and endothelial cells, we should investigate the effects of glucose, hormones and lipids on Id2 expression in these cell types as well. Additionally, it would be interesting to assess the effects of M-CSF, a well recognised proatherogenic factors, on Id2 expression. M-CSF is known to affect monocytes-macrophages differentiation. It upregulates the expression of scavenger receptor type A and CD36 expression in macrophages. It is also involved with the proliferation of macrophages in the fatty streak lesion. Its involvement in differentiation and proliferation of macrophages suggest that Id2 could be the end mediator of its effects.

Another avenue promising avenue of research is the search for novel binding partners for Id2. To date, SREBP is the only known HLH-Leucine zipper factor able to heterodimerise with Id2. Another member of the HLH-leucine zipper family which is highly involved with metabolic regulation is the Carbohydrate Responsive Element Binding Protein (ChREBP)(Uyeda, Yamashita et al. 2002). ChREBP is activated in response to high glucose and upregulates regulatory enzymes of glycolysis and lipogenesis such as L-type pyruvate kinase, phosphofructokinase, acetyle CoA carboxylase and fatty acid synthase, which are involved in lipogenesis. This factor binds to the glucose responsive element (CACGGG and CCCGTG separated by 5 bases). It contains a nuclear localisation signal, basic helix loop helix leucine zipper domain and also contains several phosphorylation sites for PKA and AMPK. At low glucose this factor is phosphorylated on ser 196 and is maintained in the cytosol. It is translocated into the nucleus when it is dephosphorylated under high glucose conditions. Phosphorylation of ser 626 and thr 666 within the bHLH-Lz domain and the DNA binding domain respectively also inhibits DNA binding by ChREBP. Because of the presence of the bHLH-Lz domain, this factor might be another target of Id2. Binding to Id2 could be another mechanism controlling its activity in addition to the control by phosphorylation.

As far as Id2 is concerned, much remains to be discovered. Nonetheless, its involvement in metabolic diseases as well as the observed therapeutic benefit from partial loss of Id2 calls for further analysis. Encouragingly, over the years small molecules inhibitors of protein-protein interaction such as those that inhibit the interaction between β-catenin-CREB have been developed (Berg 2003; Emami, Nguyen et al. 2004). If the benefit is proven, one day Id2 could become another drug target for metabolic disorders.

Chapter 7 References

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